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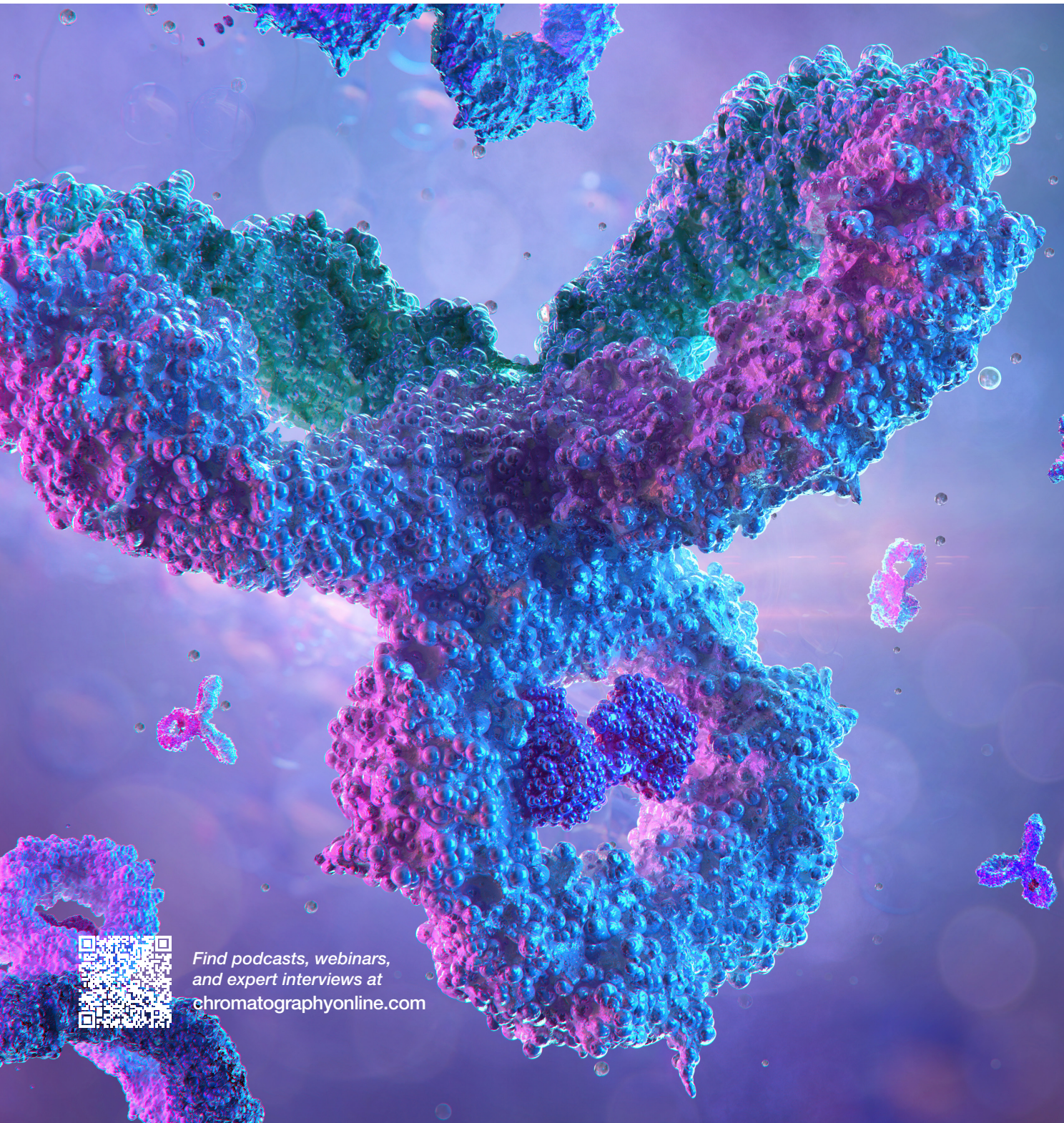

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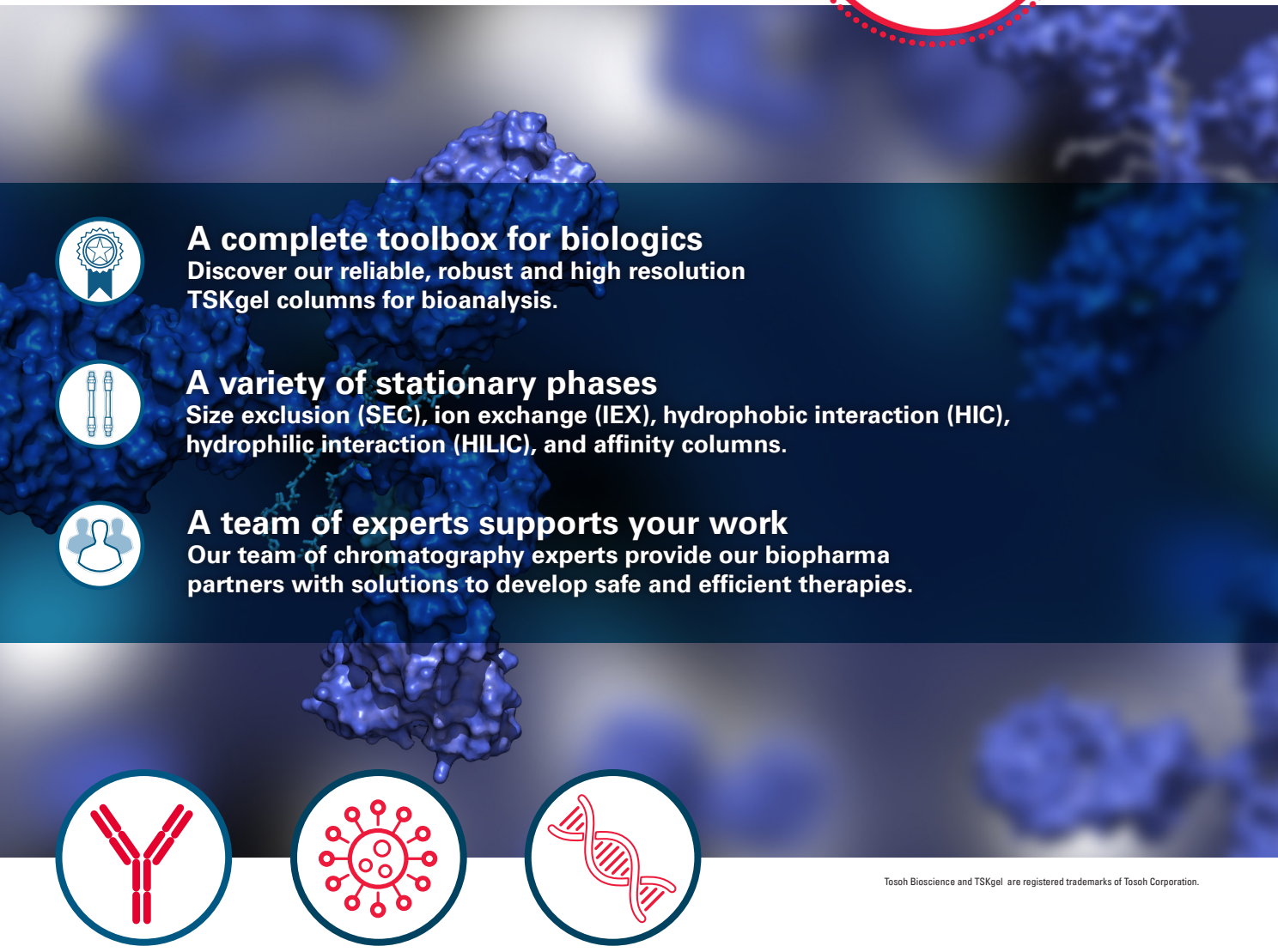

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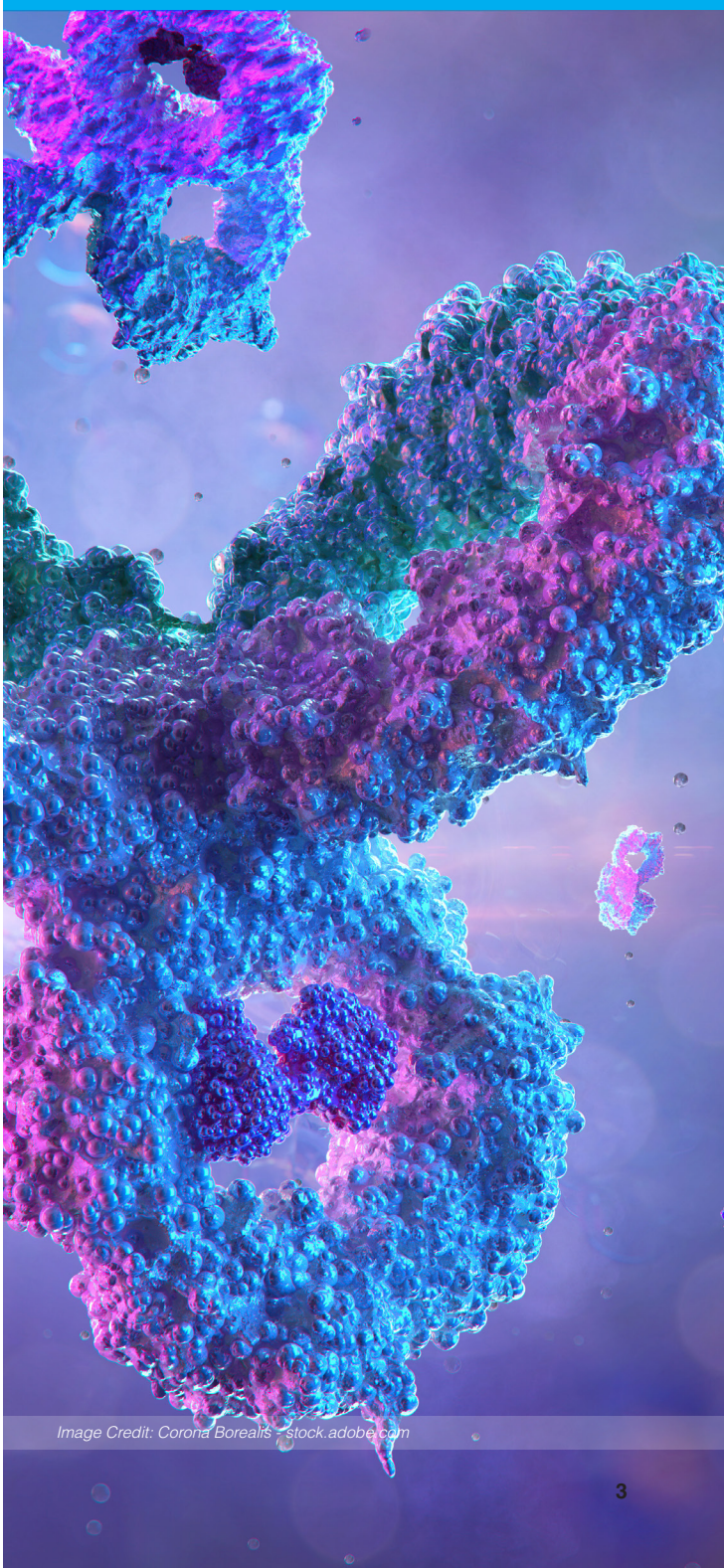

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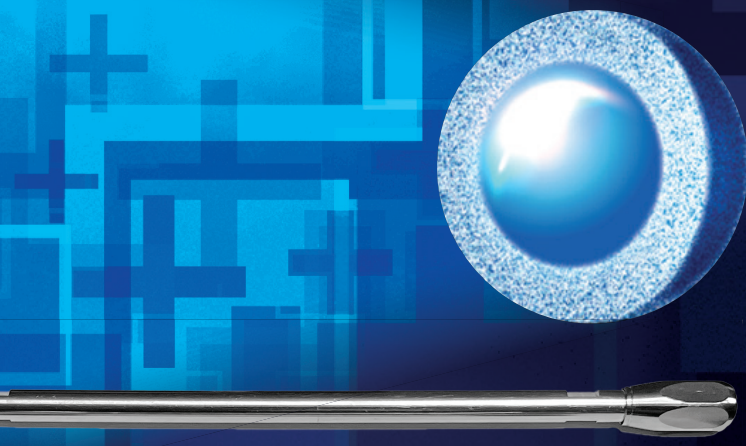

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Pushing Boundries

Guest editors, Valentina D'Atri from the University of Geneva, Switzerland and Jelle De Vos from the RIC group, Belgium introduce this special supplement in collaboration with *LCGC.*

With the advent of novel and intricate biotherapeutic formats advancing through biotech company pipelines, the significance of analytical characterization has grown exponentially. This special issue offers a comprehensive overview of contemporary analytical trends and recent advances in biopharmaceutical analysis, aiming to provide valuable insights into the analytical workflows and characterization strategies associated with biopharmaceutical products and new modalities.

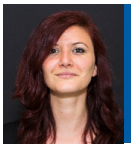
Kevin Jooß and Govert W. Somsen review analytical methods enabling the separation and identification of therapeutic proteins under native conditions, crucial for studying their higher-order structures and structure-function relationships. The review highlights the emergence of hyphenated techniques, specifically native mass spectrometry (nMS) combined with native-mode separation, as valuable tools for targeted assessment of quality attributes in biopharmaceuticals. The described methods offer insights into aspects such as aggregation, charge variants, conjugate stoichiometry, affinity, and conformation. The authors anticipate that with the further advancements in multidimensional chromatography and ion-mobility spectrometry, native hyphenated techniques will become more accessible in laboratories.

Arnaud Delobel and colleagues introduce a new chromatographic separation strategy for the analysis of therapeutic mRNA by LC–MS. The described methodologies offer a versatile and efficient characterization of mRNA quality attributes, applicable to various mRNA types without specific conditions, and integrating drug substance and product analysis. The strategy includes on-bead digestions before LC–MS characterization, enabling a comprehensive analysis of poly(A) tail, capping, residual nucleotides, and lipid characterization in lipid nanoparticles with a single sample preparation. This multi-attribute approach, requiring minimal resources, enhances time-to-result and offers flexibility for future advancs in mRNA-based therapeutic system analysis.

Anurag S. Rathore and Srishti Joshi discuss biosimilars, which are drug products closely resembling off-patent reference products in terms of purity, molecular structure, and bioactivity. The approval of biosimilars relies on demonstrating no clinically meaningful differences through analytical and functional assessments of critical quality attributes. The article specifically reviews recent biosimilarity assessments for granulocyte-colony-stimulating factor (G-CSF), insulin glargine, rituximab, and trastuzumab.

Finally, this special issue also includes an interview with Reed J. Harris, who recently retired after working for over 38 years at Genentech. In this Q&A article, Reed emphasizes the importance of chromatographic methods in detecting structural variants of monoclonal antibodies (mAbs) and identifying critical quality attributes. The interview discusses challenges in linking mAb structural changes to drug bioactivity, the significance of distinguishing different mAb size variants, and overcoming challenges in analyzing mAb charge variants. The use of multiple analytical techniques, such as isoelectric focusing and multiple attribute monitoring (MAM), is highlighted. The interview also touches on the transformative impact of mAb development on analytical scientists, career experiences, and the evolving landscape of biotechnology, including new modalities like gene therapy products.

This special issue focuses on diverse methodologies to address challenges in the analytical characterization of biotherapeutics within a dynamic and complex landscape. All contributions showcase the necessity for analytical workflows and characterization strategies to adapt to the evolving landscape of next-generation biotherapeutic formats. We hope that these latest insights may inspire scientists to not hold back and keep pushing the boundaries of separation science and mass spectrometry.



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Native Separation-Mass Spectrometry in Biopharmaceutical Analysis

Kevin Jooß and Govert W. Somsen, [a] Division of Bioanalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, Amsterdam Institute of Molecular and Life Sciences, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands and [b]Centre for Analytical Sciences Amsterdam (CASA) The Netherlands.

Analytical methods that allow separation and identification of therapeutic proteins under native conditions play a crucial role in studying their higher-order structures and structure–function relationships. Recently, hyphenated techniques that combine native-mode separation with native mass spectrometry (nMS) have emerged as highly valuable tools for the targeted assessment of these quality attributes. This article outlines current native separation strategies coupled with nMS designed to characterize biopharmaceuticals close to their natural state. The methods provide worthful insights into aspects like aggregation, charge variants, conjugate stoichiometry, affinity, and conformation. As multidimensional chromatographic techniques and ion-mobility spectrometry become more accessible in laboratories, further advances in the development of native hyphenated techniques capable of simultaneously providing compositional, structural, and functional information on biopharmaceuticals can be expected.

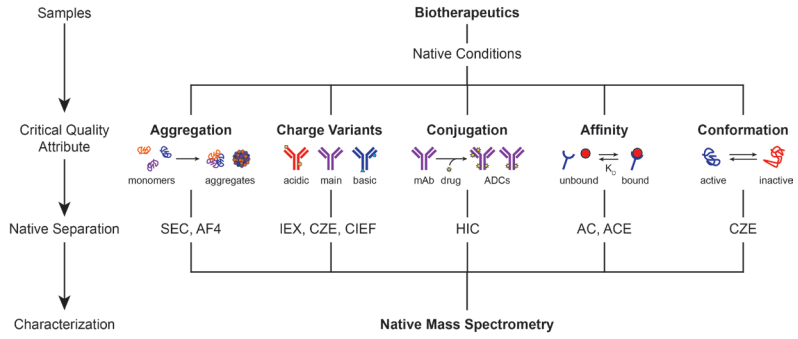
Over the past few decades, there has been substantial progress in the development of mass spectrometry-based tools for the structural analysis of intact proteins, driven by requirements in the fields of biopharmaceuticals, structural biology, and top-down proteomics. Proteins, as entities, frequently exhibit considerable heterogeneity reflecting a plethora of subtle structural modifications.

The characterization of these different proteoforms is a challenging but frequently indispensable task. For example, during development and production of therapeutic proteins, it is crucial to monitor several critical quality attributes (CQAs) that are linked to the presence of protein variants (1). In addition to chemical variations, factors such as conformational and supramolecular structures (including aggregates, conformers, and complexes), as well as the level of bioactivity, can contribute to the heterogeneity of biopharmaceuticals. Evaluating these properties requires the preservation of the natural conformation and functional state of the proteins of interest throughout the analysis process.

Native mass spectrometry (nMS) has been demonstrated to be a highly useful approach for gaining deeper understanding of the structure and behavior of proteins in conditions closely resembling their natural or native state (2). In this context, native reflects a protein's biological status prior to ionization. This involves the protein being immersed in an aqueous solution with a pH and ionic strength conducive to preserving its natural folded conformation (3). nMS provides accurate mass determinations of intact proteins by employing electrospray ionization (ESI) that, in principle, can preserve conformation and non-covalent interactions of proteins in the gas phase (4). Consequently, nMS allows for the analysis of tertiary and quaternary structures, protein–substrate and protein–protein complexes, related stoichiometries and binding affinities, and even large macromolecular assemblies. When proteins are sprayed under native conditions, for example, using 100 mM ammonium acetate (AmAc) at pH 7 as solvent, they display charge state distributions (CSDs) that are distinctly different from those obtained under conventional, denaturing conditions: a reduced number of charge states, resulting in considerably narrower CSDs, and a decrease in overall charge, shifting CSDs to the higher m/z region. The nature of a protein's CSD can indicate whether, and to what extent, a protein is in its native conformation.

To obtain high-quality and information-rich protein mass spectra with nMS, it is necessary to work with purified samples (5). Extensive sample pretreatment is frequently required, particularly when the proteins of interest are part of complex matrices/samples. Furthermore,

FIGURE 1: Overview of critical quality attributes of therapeutic proteins addressed by the online combination of native separation modes with native mass spectrometry.



it is crucial to exchange nonvolatile buffers and salts in the sample for volatile substances, commonly AmAc, to ensure MS compatibility. As a result, the necessary sample preparation is often time-consuming and labor-intensive, with the added risk of inducing protein modifications, degradation, or denaturation. The burden on sample preparation can be alleviated by incorporating efficient analytical separation prior to nMS detection. Hyphenation of protein separation techniques to nMS can strongly reduce the complexity of obtained mass spectra, facilitating the detection of low abundant protein species or proteoforms with subtle mass differences, up to the distinction of isomeric species. Additionally, analytical separations intrinsically encompass sample cleanup and the possibility of buffer exchange, circumventing ion suppression and unwanted adduct formations. Upfront separation helps filter out protein species formed as artefacts during the ionization process. For example, although intrinsically a soft ionization method, ESI can induce protein aggregate formation, particularly at high protein concentrations. Coupling size-based separation to nMS allows to differentiate between aggregates that genuinely exist in the sample and those that are merely false aggregates generated by the ESI process.

When the goal is to characterize higher-order structures or protein affinity by nMS, the hyphenated separation methods should preserve natural protein conformations and interactions. This requirement *a priori* excludes the commonly applied protein separation techniques reversed-phase liquid chromatography (RPLC) and hydrophilic interaction chromatography (HILIC), which by default use denaturing organic solvents in their eluents. When conducted aptly, which means using aqueous mobile phases of proper pH, ionic strength and temperature, separation techniques such as size-exclusion chromatography (SEC), ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), affinity chromatography (AC), and asymmetrical flow field flow fractionation (AF4) can, in principle, offer valuable protein resolutions while maintaining native conditions (6,7). In addition, electromigration-based techniques such as capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), and affinity capillary electrophoresis (ACE) can be conducted under native conditions achieving high separation efficiencies (8). Nevertheless, when directly coupled with nMS, adjustments to routinely used methods are necessary, as these often employ phosphate buffers or salt gradients that are not compatible with MS (9).

The objective of this paper is to provide a concise overview of the available online combinations of native separation and nMS, and how they can yield distinctive and exceptionally valuable information regarding a range of crucial properties in biopharmaceutical analysis. The topics covered are characterization of aggregates, charge variants, conjugates, affinity, and conformation of protein therapeutics (Figure 1). Each subject is introduced followed by typical examples that demonstrate the performance, applicability, and scope of the respective native techniques in more detail. More comprehensive overviews on the hyphenation of native protein separations with MS can be found elsewhere (6,8,10).

Protein Aggregates

Characterization of the size variants of therapeutic proteins is essential to ensure the quality and safety of biopharmaceutical products. The presence of protein fragments and subunits is indicative for bioprocess performance and protein stability, whereas protein aggregates, such as dimers, can be highly immunogenic. Size-based separation techniques, such as SEC and AF4, can resolve proteins from their aggregates and fragments under native elution conditions. These circumstances are critical as most aggregates are held together by non-covalent interactions, which may be particularly sensitive to denaturing environments. The coupling of SEC and AF4 to nMS facilitates the identification of size variants while also providing information on other structural aspects, including post-translational modifications (PTMs).

SEC–nMS: Native SEC for biopharmaceutical analysis is commonly carried out using aqueous eluents

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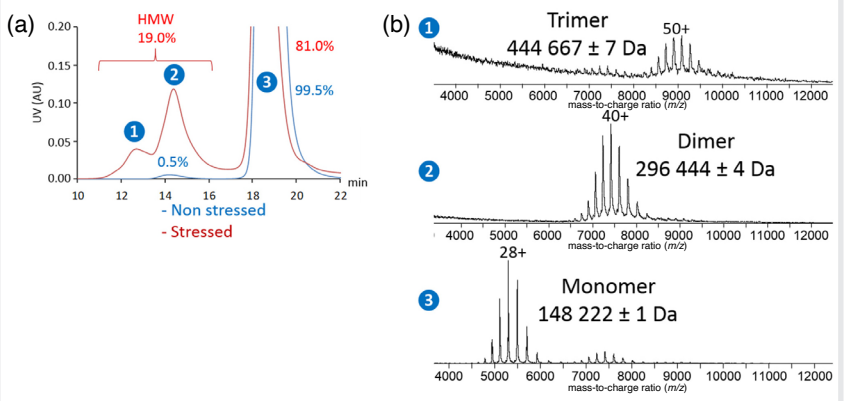
FIGURE 2: SEC–nMS of trastuzumab. (a) Overlaid chromatograms of pH-stressed (red trace) and unstressed (blue trace) trastuzumab; high-molecular-weight (HMW) variants; Percentages indicate relative peak areas. (b) Native mass spectra of corresponding peaks in chromatogram of stressed tratuzumab. Reprinted and adapted with permission from reference (11).



containing non-volatile buffer salts at substantial concentrations. When coupling SEC to nMS, it becomes necessary to substitute these for volatile, MS-compatible, electrolytes. Using a mobile phase containing 100 mM AmAc (pH 6.8), SEC–nMS has demonstrated its ability to both identify and quantify side- and degradation products of pharmaceutical monoclonal antibodies (mAbs) (Figure 2) (11). Along similar lines, Haberger et al. used SEC–nMS to profile bispecific antibody samples for product-related impurities derived from incorrect light or heavy chain association during production, and aggregates, formed during storage and exposure to elevated temperature (12). Employing short columns of less than 5 cm filled with narrow-pore particles, SEC has been integrated with nMS to enable rapid and automated desalting of samples, without attaining protein separation (13). This concept has been utilized in online two-dimensional (2D) LC schemes to combine native protein separations employing non-volatile salts with nMS. Fractions from MS-incompatible SEC modes were comprehensively analyzed by native short-column SEC using volatile eluents, allowing structural assessment of mAb size variants when coupled to nMS (9). In SEC employing long columns (15–30 cm) filled with wide-pore particles, the use of volatile mobile phases can result in compromised SEC performance, for example, distorted peaks and shifts in retention time due to unintended interactions with the SEC column and the stationary phase material. These interactions may also cause partial protein unfolding. Ventouri et al. demonstrated that when it comes to retaining native protein conformations in long-column SEC–nMS, AmAc is the most effective among options such as ammonium formate and bicarbonate, however, only when used at appreciable ionic strength (200 mM) (14). Such concentrations can still induce protein ionization suppression and in combination with common SEC flow rates requires relatively harsh ESI conditions, which potentially disrupt protein higher-order structures. This limitation can be alleviated, at least to some extent, by employing innovative bioinert SEC columns featuring chemically modified surfaces, which reduce unwanted protein interactions. This is illustrated by Murisier and colleagues in their work on size-variant analysis of various mAbs through SEC–nMS, using an eluent containing 50 mM ammonium acetate (15). An alternative solution lies in adopting micro-flow SEC—nMS, using 1.0 mm i.d columns operated at 15 µL/min. This approach significantly improves ionization efficiency allowing use of concentrations as high as 400 mM AmAc to prevent protein interactions with conventional SEC stationary phases (16). The effectiveness of this approach was demonstrated by the analysis of fragments and aggregates of trastuzumab and the tetrameric anticancer drug asparaginase (ASNase).

AF4–nMS: AF4 is a separation technique suitable for resolving large (bio)macromolecules, aggregates, and particles based on their size (17). The underlying separation mechanism relies on differential diffusion of analytes in a flow field perpendicular to the laminar flow of a carrier liquid in a thin open channel. Protein samples can be fractionated by size without the use of a stationary phase, excluding any adverse protein interactions with packing material. This allows for the use of MS-compatible mobile phases comprising relatively low concentrations (10-20 mM) of AmAc without losing separation performance. In addition, AF4 circumvents the potential physical stress exerted on protein structures while passing through the narrow channels of columns packed with stationary phase particles (18). These attributes are particularly valuable for the quality and stability assessment of relatively labile biopharmaceuticals, such as ASNase, where the tetramer serves as the actual active pharmaceutical compound. The AF4–nMS analysis of an unstressed ASNase sample revealed the presence of both octamer and monomer species next to the drug substance (19). It also unveiled the gas-phase dissociation of tetramer into monomer and association

of tetramer to octamer during ESI, which would go undetected if direct nMS without prior separation was used.

Protein Charge Variants

Presence of charge variants is one of the key CQAs of biopharmaceuticals, in particular for mAbs (20). The typical approach for characterizing protein charge profiles involves techniques such as ion exchange chromatography (IEX), capillary zone electrophoresis (CZE), and imaging capillary isoelectric focusing (iCIEF) in combination with UV-Vis absorbance detection. While these methods are efficient in a quality control setting, they do not allow unambiguous structural assignment of expected and unexpected peaks. Coupling with MS, however, represents a challenge, mainly due to the common use of non-volatile buffer or mobile phase components to achieve optimal separation performance. In recent years, native-based separation techniques in combination with nMS, have emerged as a solution for charge variant analysis (CVA). There are two primary advantages associated with using nMS for CVA. Firstly, there are fewer interfering ions in the higher mass-to-charge ratio *(m/z)* region. Secondly, proteoforms carrying different modifications are better resolved due to the wider *m/z* spacing among proteoforms at lower charge states.

IEX–nMS: Strong cation exchange chromatography (SCX) is a technique that separates protein species based on their surface charge. In general, SCX separation can be carried out under native conditions (21). Yan et al. outlined the coupling of SCX with nMS, employing a combined pH (5.6–7.4) and salt (20–150 mM) gradient for the CVA of various mAbs spanning a wide pI range. This approach allowed for the detection of minor modifications such as a non-consensus Fab glycosylation site (<0.1%) in the NIST mAb reference standard. Haberger and colleagues developed a SCX-MS method using a pH gradient ranging from 6.0 to 10.7 for the CVA of a 200 kDa T-cell bispecific humanized mAb, containing an additional Fab domain. With this approach, they not only identified a new N-glycosylation motif in the heavy chain CDR region, but also separated glycoforms carrying varying degrees of sialylation (22).

CZE–nMS: In CZE, proteins are separated in a fused-silica capillary filled with a background electrolyte (BGE) solution under the influence of an electric field. The electrophoretic mobility of a protein is proportional to the ratio of its charge and hydrodynamic radius. This property makes CZE exceptionally proficient in separating proteoforms of similar size, but different in the nature and number of charge-inducing PTMs, such as sialylation, deamidation, pyroglutamate formation and lysine clipping (23). The resolution of proteoforms is generally enhanced when the pH of the BGE is close to the isoelectric points (pIs) of the target molecules. Consequently, various CZE–UV methods for CVA use BGEs in the pH range of 5 to 7, particularly for mAb characterization. CZE–MS of intact proteins, including profiling of pharmaceutical (glyco)proteins, has been demonstrated, but frequently using acidic, denaturing conditions (24). An auspicious configuration for coupling CZE to nMS involves using a microfluidic device equipped with an integrated spray tip designed for direct electrospray ionization from the separation channel (25). Using native conditions, Carillo et al. demonstrated the effectiveness of microCZE–nMS for separating a diverse set of mAbs, including rituximab, trastuzumab, and bevacizumab, using a commercially available BGE kit (26). They achieved remarkable resolution between various charge variants, including basic proteoforms carrying non-processed C-terminal lysine, non-cyclized N-terminal glutamate, and succinimide, as well as acidic proteoforms exhibiting deamidation and sialylation (Figure 3). Wu and colleagues used a micro-CZE–nMS set up expanding the field of application to bispecific mAbs as well as therapeutic antibodies in the "N±1" format, where either

FIGURE 3: CZE–nMS of rituximab using a microfluidic chip platform. (a) Total-ion electropherogram with assignment of the separated basic and acidic charge variants based on observed mass. (b) Mass spectrum of the parent mAb with zoom of the most abundant charge state. Reprinted and adapted with permission from reference (26).

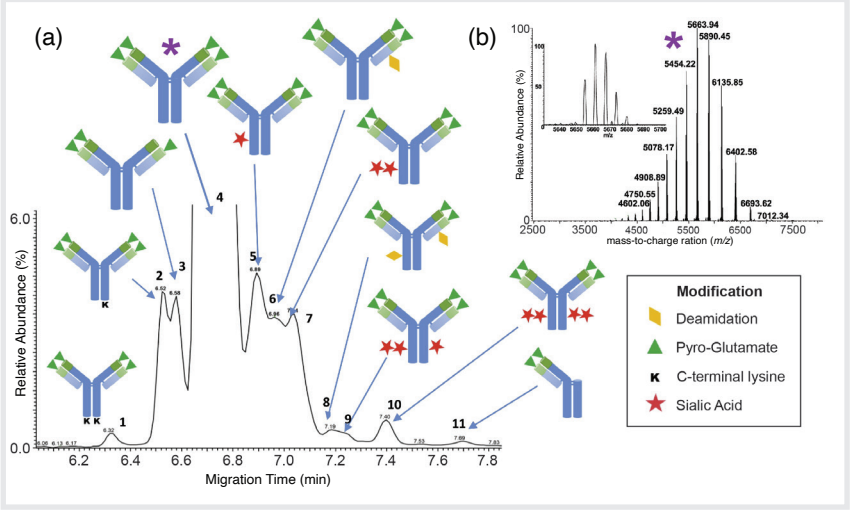
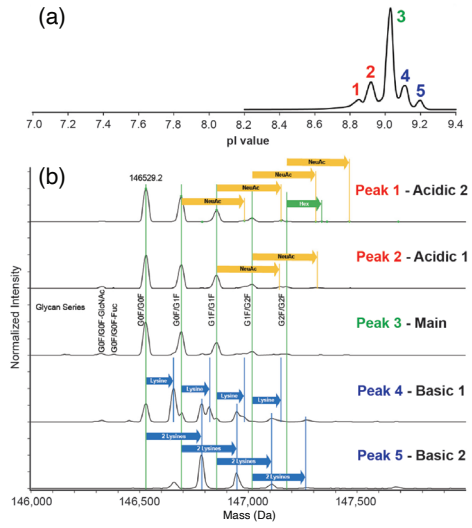


FIGURE 4: Imaged CIEF–nMS of an IgG2 antibody (mAb4). (a) Imaged CIEF–UV trace of mAb4. (b) Deconvoluted mass spectra of mAb4 (peak 3) and its acidic (peak 1 and 2) and basic (peak 4 and 5) variants. The acidic and basic variants appear to contain sialic acids and unclipped C-terminal lysines, respectively. Reprinted and adapted with permission from reference (31).



one Fab arm is removed or an additional one was added to the mAb construct (27).

CIEF–nMS: CIEF is a technique that separates proteins according to their pI value utilizing a pH gradient. Charge-inducing PTMs noticeably impact the pI of a protein. Consequently, CIEF proves to be effective for resolving charge variants. Although CIEF, like CZE and IEX, relies on protein charge for separation, the selectivity may differ, leading to varying degrees of resolution based on the nature of the charge variants. The pH gradient is formed using a mixture of ampholytes, molecules containing both acidic and basic functional groups, defining the pH gradient range. Effective online hyphenation with MS remains a challenge, due to the ion suppression caused by ampholytes. One approach to reduce ion suppression is to directly couple CIEF to MS detection using substantially reduced amounts of ampholytes in the CIEF BGE, which will compromise protein resolution. Dai and colleagues used a nano-sheath flow-based CIEF–MS setup for the characterization of mAb charge variants, employing 1.5% v/v Pharmalyte 3-10 with 5 – 20% glycerol in the sample mixture (28). Glycerol helped to attain a stable and linear pH gradient along the migration path. Due to the application of 25 – 50% acetonitrile in the sheath liquid for CE–MS interfacing, denatured mass spectra of the mAb proteoforms were detected. Przybylski et al. characterized cytokine human interferon-gamma using CIEF–nMS by using 10 mM AmAc (pH 5) as sheath liquid (29). Typical native-like mass spectra were observed. Recently, one of the first microfluidic chip-based imaged CIEF–MS systems was launched at the 71st ASMS conference. The system allows monitoring of the mAb charge profile during the focusing step, followed by subsequent mobilization, and detection by MS. Notably, relatively high ampholyte concentrations (4% v/v) can be employed, without a distinct impact on MS sensitivity (30). Recently, the field of applications was expanded to different IgG subclasses, covering a wide range of pI values (7.3 – 9.0), and an illustrative example of the CVA of a IgG2 mAb is depicted in Figure 4 (31).

Protein Conjugates

Antibody-drug conjugates (ADCs) are produced by covalently coupling low-molecular-weight drugs to mAbs via cleavable linkers, resulting in a distribution of conjugates, typically ranging from 0 to 8 drug molecules for each mAb. The average drug-to-antibody ratio (DAR) is an essential CQA which needs to be carefully assessed. HIC has proven to be particularly valuable for DAR analysis of ADCs in which the drugs are linked to interchain cysteine residues (32). These so-called Cys-ADCs exhibit decreased structural stability compared to the parent mAb, due to the reduction of several disulfide bridges to accommodate drug attachment. The capacity of HIC to maintain native-like conditions allows to preserve the antibody structural integrity while achieving efficient conjugate separation. Online coupling of HIC to nMS is particularly intriguing in this context because it can provide accurate assignment of the respective conjugates and potential characterization of unexpected byproducts. HIC typically relies on gradients of non-volatile sulfate and phosphate salts. Substituting these with volatile alternatives is challenging, as conjugate separations are either strongly compromised or require AmAc concentrations incompatible with direct MS detection. To overcome this obstacle, Ehkirch et al. adopted a multidimensional strategy (HIC–SEC-MS) in which short-column native SEC was added as second LC dimension to attain salt exchange and MS compatibility (33). Using both untreated and artificially degraded brentuximab vedotin as model ADC, the approach allowed unambiguous profiling of the conjugate composition, including DAR. Moreover, it had the capability to

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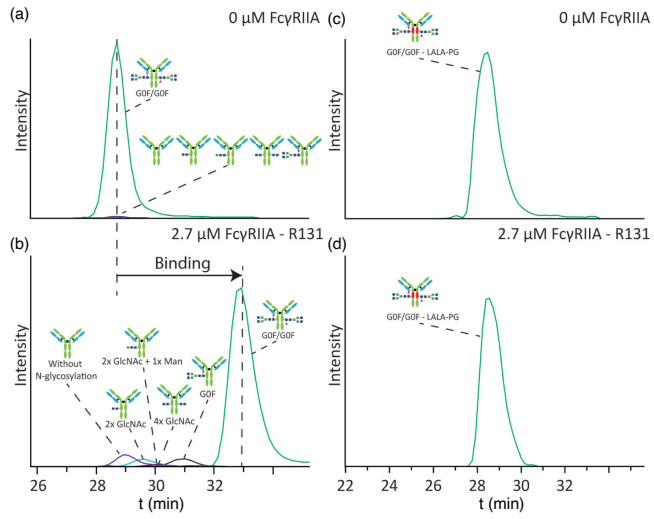
FIGURE 5: ACE–nMS of mAb-A probing the binding to the FcγRIIa receptor as a function of Fc N-glycan occupancy. (a and b) Extracted-ion electropherograms of mAb-A glycoforms recorded with (a) 0 µM or (b) 2.7 µM FcγRIIa R131 variant present in the BGE. (c and d) Extracted-ion electropherograms of the glycoforms of an Fc-silencing mutant of mAb-A recorded with (c) 0 µM or (d) 2.7 µM FcγRIIa R131 variant present in the BGE. Reprinted with permission from reference (42).



characterize potential positional isomers, ADCs with non-standard DARs, and degradation products. Yan and co-workers proposed an alternative strategy to achieve HIC–nMS, involving online post-column dilution (1:6, v/v) of the 3.0-0.3 M AmAc HIC gradient with water followed by flow splitting (about 500:1), ultimately directing a flow of 1-5 µL/min to ESI-nMS (34). The utility of this approach was demonstrated by characterizing a Cys-ADC mimic under native conditions, revealing positional isomers and degraded species next to the expected conjugates. In addition, this method proved to be useful for disclosing the presence of O-linked glycan and oxidation variants in mAb samples.

Protein Affinity

Evaluating the quality and characteristics of interactions between proteoforms of a biopharmaceutical and their dedicated target is critical in drug development to understand and ensure efficacy of the product. Traditional assays for assessing affinity and binding properties typically yield a single, averaged response for a protein species (35). However, these methods do not offer the capability to differentiate between proteoforms or discern the influence of related variants on protein affinity. The combination of native protein separation with nMS can enable the investigation of structure-function and binding relationships at the proteoform level.

AC–nMS: In AC-based approaches, ligands or receptors are typically immobilized on a stationary phase (36). The retention of proteoforms is determined by their binding affinity with their respective target. AC plays a vital role in protein purification, but it can also be applied for determining protein binding characteristics. In AC, the initial mobile phase is designed to mimic physiological conditions, facilitating relevant binding interactions with the immobilized ligands or receptors, all while striving to reduce non-specific binding to a minimum. Gradually changing the pH or ionic strength of the mobile phase disrupts the interactions, eluting the proteoforms with respect to their affinity. The capabilities of AC-nMS for biopharmaceutical analysis were demonstrated through the analysis of the association of mAb proteoforms with fetal/neonatal Fc receptors (FcRn) as well as FcγRIIIa (37, 38). In both cases, the receptors were immobilized on a Sepharose stationary phase, and elution was performed applying a pH gradient. This allowed for the selective distinction of proteoforms exhibiting methionine oxidation, a common degradation, in the Fc binding domain, (37) and the influence of mAb glycosylation type on FcγRIIIa binding (38). Despite these promising results, AC–nMS faces certain limitations, including the injection of a relatively large amounts of mAb (≥50 µg), the limited pressure-resistance of AC columns, and the large amounts of receptors required to produce AC columns. These challenges might be overcome through miniaturization, potentially down to the microchip level.

ACE–nMS: ACE in combination with nMS is an auspicious approach to study non-covalent protein interactions providing proteoform resolution and identification. In ACE–MS, the ligand of interest is added to a non-denaturing BGE, and the binding receptor is injected as sample or vice versa (39). When proteoforms interact with the free ligand or receptors during CE analysis, they undergo a shift in their electrophoretic mobility. This shift is a result of the difference in charge-to-size ratio between the protein complex and the unbound protein. The extent of this shift is governed by the affinity constant (Kd), which conceptually can be determined for each of the separated proteoform species. Unlike AC approaches, ACE does not require specific column material and only limited amount of receptor and ligand, making it more feasible

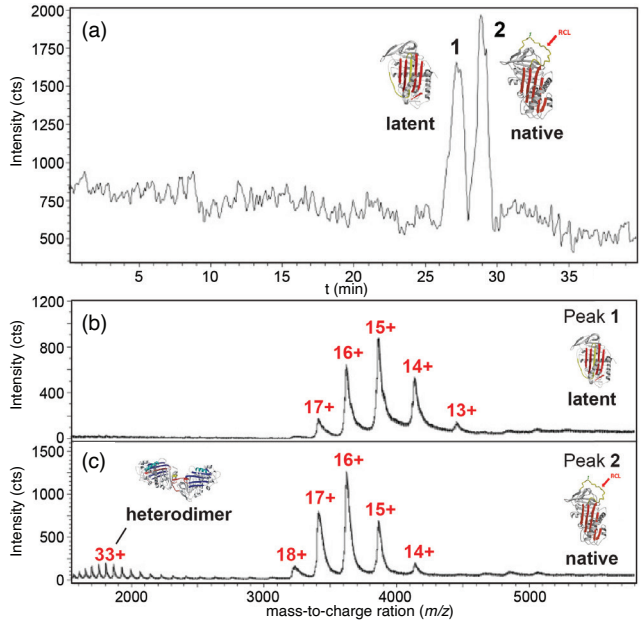
for screening a variety of receptors and ligands. Additionally, ACE avoids unwanted changes in binding properties resulting from immobilization reactions (40). Conventional ACE–UV is conducted using non-volatile buffers such as sodium phosphate. In ACE–MS, these buffers are typically substituted with ammonium acetate- or bicarbonate-based BGEs at near-physiological pH. The Domínguez-Vega group utilized ACE–nMS to conduct functional studies on the binding of mAbs to FcRn (41). This approach enabled them to determine binding affinities at the individual proteoform level. Their findings revealed a reduction in mAb binding affinity in response to oxidative stress, as also indicated by a higher Kd value. Additionally, variations in glycosylation led to minor shifts in migration time, signifying that proteoforms carrying larger glycans displayed stronger binding to FcRn. The same group also investigated the binding of different mAb glycoforms to the FcγRIIa receptor by ACE-nMS (Figure 5) (42). For a mAb, exhibiting two, one, or no N-glycosylations showed that the binding affinity substantially diminished with decreasing glycosylation (Figures 5a and 5b). To confirm that the observed changes in electrophoretic mobility are indeed associated with specific receptor binding, they also examined a mutant mAb with three amino acid substitutions, resulting in silencing of the Fc region. Indeed, virtually no binding was observed between the glycoforms of the mutant mAb and FcγRIIa (Figures 5c and 5d). In addition, they unveiled, for the first time, that high mannose glycoforms exhibit diminished affinity for FcγRIIa.

Protein Conformation

In contrast to small-molecule drugs, the conformational characteristics of protein biopharmaceuticals in solution are affected by a range of factors that go beyond their covalent chemical structures. As the conformation of a protein is closely tied to its biological function, the capacity to identify alterations is crucial in the development, purification, and formulation of a biopharmaceutical that needs to maintain consistent therapeutic properties (43).

Conformational changes of proteins do not only impact protein shape but can also have a distinct influence on net charge. In this respect, the charge-to-size separation mechanism of CZE appears eminently suitable to probe protein conformational changes under native conditions (44). In a biopharmaceutical context, CZE–MS has been used to differentiate between conformers of antithrombin (AT), a biopharmaceutical product employed for the treatment of hereditary or acquired AT deficiencies (45). Employing a BGE of 50 mM ammonium acetate (pH 7.4), CZE provided separation of the natural, active form of AT from the latent, conformationally more compact form, which exhibits no activity (Figure 6). The isomeric AT forms could be assigned as the CSD in the recorded mass spectrum of the latent form was shifted to higher *m/z* with respect to the active form. The CZE–MS method allowed tracking of latent form formation in AT preparations.

FIGURE 6: CZE–MS of native and latent forms of antithrombin. (a) Base peak electropherogram (1500–5000 *m/z*); (b) mass spectrum of peak 1 (latent form); (c) mass spectrum of peak 2 (native form). Reprinted and adapted with permission from reference (45).



Conclusion

Online combinations of native protein separations with nMS have recently clearly proven to be powerful and unique means for the structural and functional characterization of biopharmaceuticals. Practical solutions have been developed to enhance the MS compatibility of native separation techniques, among which are several multidimensional approaches. 2D-LC equipment has become commercially available providing not only online desalting options, but potentially also increased resolving power

by employing complementary separation selectivity. Furthermore, advancements in high-resolution MS instrumentation have made it easier to detect intact proteins in their natural state. The direct benefits of hyphenating nMS with native separation lie in the associated reduction of sample complexity, sample cleanup, and buffer exchange, ultimately resulting in protein mass spectra that yield higher-quality information. In addition, there are evident synergistic advantages to this combination. Upfront separation can facilitate deconvoluting MS signals into distinct contributing species. Conversely, the mass spectrum of a single peak may reveal the presence of multiple species, such as structural variants, aggregates, isomers, or even conformers. In the latter respect, the incorporation of ion-mobility (IM) spectrometry into native separation-nMS workflows will greatly broaden their capabilities. Over the last two decades, native IM-MS has emerged as a highly valuable tool in structural biology for the study of protein conformation and aggregation, (46) and its potential role in biopharmaceutical analysis has been recognized (47). The possibility of hyphenating native SEC and HIC to IM-MS has been demonstrated a few times, however, protein conformational information has yet to be extracted from the IM dimension (33). In coming years, there will be an expansion of the analytical toolbox with native hyphenated approaches, undoubtedly enabling more in-depth characterization of biopharmaceuticals in a shorter timeframe.

Conflicts of interest

The authors declare no conflict of interest.

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Multi-Attribute Monitoring of Therapeutic mRNA by Liquid Chromatography–Mass Spectrometry

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Therapeutic mRNA is receiving growing interest in various therapeutic applications such as genome editing, cancer immunotherapy and prophylactic vaccines. As with other drugs, it is essential to guarantee product quality. Among the critical quality attributes of therapeutic mRNA, characterization of the capping and poly(A) tail are of the greatest importance because of their involvement in mRNA stability and in the efficiency of protein synthesis. This article presents a method for the simultaneous characterization of both attributes in a single sample preparation workflow. The method involves lipid extraction, various RNAse enzymes, purification steps and LC–MS to analyze the capping and poly(A) tailing.

Therapeutic messenger RNA (mRNA) have emerged as a game-changer in medical science (1), offering transformative approaches in the creation of prophylactic vaccines and gene therapies. Unlike traditional vaccines, which use attenuated viruses or antigenic proteins to elicit immune responses, mRNA-based vaccines deploy sequences encoding specific antigens to induce robust immunological defence against targeted pathogens. This approach not only streamlines vaccine development but also enables rapid adaptation to emerging epidemics and mutated virus variants (2). Beyond vaccines, the utility of therapeutic mRNA extends to gene therapy (3), providing a strategy to correct or replace defective genes associated with genetic disorders, and in innovative approaches to treat certain cancers. Therapeutic mRNAs are commonly encapsulated in lipid nanoparticles to facilitate their delivery to cellular ribosomes, where they are translated into polypeptide sequences. Structurally, a mRNA molecule consists of a central open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTRs). Critical to mRNA functionality are two terminal features: the 5'-cap and the 3'-poly(A) tail.

The 5'-cap serves multiple roles, including the protection of mRNA from exonuclease-mediated degradation and facilitation of ribosomal binding during translation initiation. This cap is primarily composed of 7-methylguanosine (m7G) connected via a 5' to 5' triphosphate linkage to the mRNA's first nucleotide. Depending on the methylation status of this first nucleotide, two distinct structures—Cap0 (unmethylated) and Cap1 (methylated)—may form. Cap1 mRNA, which is the expected structure, can be produced through either post-transcriptional enzymatic capping, in which the cap is added through several enzymatic reactions after the transcription reaction, or co-transcriptional incorporation of a cap analog (4).

The 3' poly(A) tail, a series of adenine nucleotides, protects mRNA from degradation by exonucleases and improves its translation efficacy by interacting with key proteins in the translation machinery (5). This tail can be either directly encoded in the DNA template for *in vitro* transcription or enzymatically added post-transcription using poly(A) polymerase (6).

Given their importance for obtaining a fully functional therapeutic mRNA, capping efficiency, that is, the percentage of Cap1 structure obtained after *in vitro* transcription and poly(A) tail length distribution are two critical quality attributes (CQAs) for mRNA drug substance purity (7). Sequencing approaches could be used to obtain poly(A) tail length information using methods such as TAIL-seq, PAL-seq, or RT-PCR but they are not recommended for this purpose by USP (8,9). While liquid chromatography methods to analyze these CQAs exist, they often require specific primers for hybridization and rely on RNAse H (10,11), requiring adjustments for each cap and poly(A) tail to be analyzed and lacking a universal platform for assessment. Recently, Gilar et al. released a method based on RNAse T1 digestion and SEC or RP separation

of the digests obtained (12). Traditional quality assessment often demands substantial sample volumes and extensive use of instruments. The development of rapid, innovative techniques requiring limited sample amounts is crucial. Such advancements not only preserve material but also free up mass spectrometer time, yielding significant operational benefits and cost reductions. By providing results on multiple quality attributes swiftly, these methods hold the promise to markedly enhance the quality assessment process, meeting the growing demands of various industries and regulatory standards.

In this study, we introduce optimised methodologies for the comprehensive characterisation of poly(A) tail length and capping structures. By refining sample preparation protocols, a single workflow has been developed that allows simultaneous assessment of both CQAs, thereby minimizing sample usage and reducing experimental time. This approach leverages a combination of multiple RNases, a purification step, and liquid chromatography–mass spectrometry (LC–MS) for detailed analysis. Additionally, an optimized extraction step has been incorporated to facilitate the characterization of lipid formulations in drug products.

FIGURE 1: Capping identification and relative quantification for Cas9 mRNA. (a) UV chromatogram of Nuclease P1 digest. (b) Zoom on Cap elution region. (c) Relative quantification of cap.

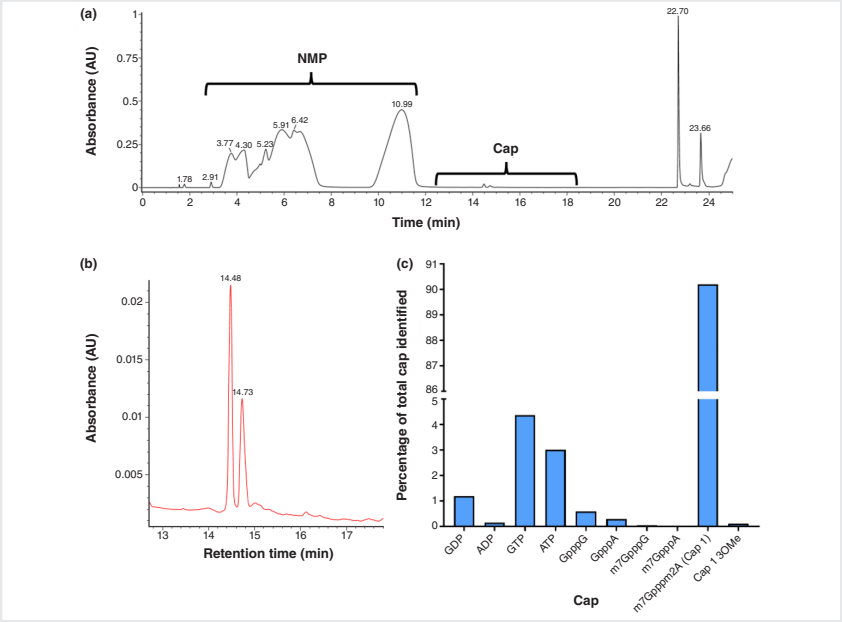
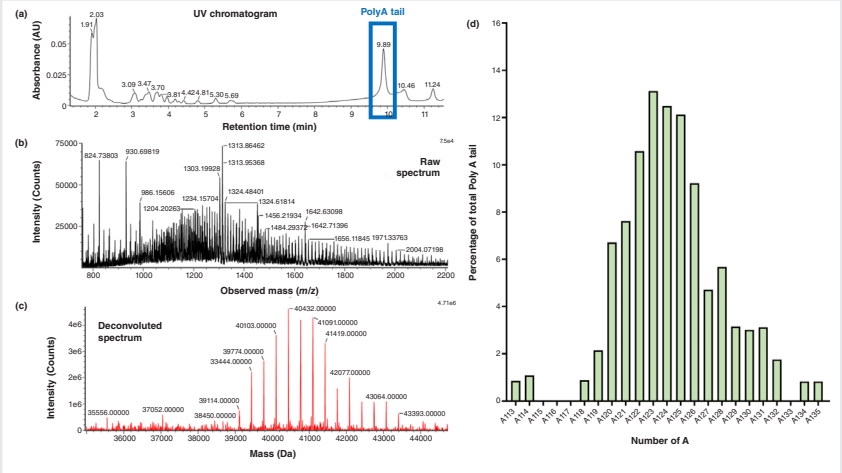


FIGURE 2: PolyA tailing distribution for Cas9 mRNA. (a) UV chromatogram of RNAse T1 digest. (b) Raw mass spectrum of polyA tail peak. (c) Deconvoluted mass spectrum of polyA tail peak. (d) Size and abundancy distribution of polyA tails.



Materials and Methods

Chemicals and Reagents: Isopropanol, ammonium acetate, ADP, ATP, GDP, GTP, dibutylamine, dibutylammonium acetate, and formic acid were purchased from Sigma Aldrich. Acetonitrile, methanol, and hexafluoroisopropanol were purchased from Biosolve. RNA purification kit (61006), RNAse T1 (1000 U/µL) and RNAse free water were purchased from Thermo Scientific. Nuclease P1 (100 U/µL) and several cap (GG, GA, m7GG, m7GA, ARCA) were obtained from New England Biolabs. CleanCap Reagent AG (N-7113) as well as Cas9 mRNA (used for both method development and as drug substance – L7206) were purchased from TriLink BioTechnologies. LNP-encapsulated mRNA was provided by collaborators (undisclosed composition).

Extraction of mRNA from Drug Product: mRNA is extracted using isopropanol precipitation. 20 µg mRNA equivalent of LNP-encapsulated mRNA are diluted in 900 µL 60 mM ammonium acetate in isopropanol, vortexed thoroughly and centrifuged 15 min at 14,000 g. Supernatant is collected for lipid analysis. The pellet is mixed with 1 mL of

FIGURE 3: Residuals and capping identification and polyA tail distribution for Cas9 mRNA after purification. (a) Workflow of the multiple attribute monitoring sample preparation and analysis of mRNA drug substance. (b) Relative quantification of residuals. (c) Relative quantification of cap. (d) Size and abundancy distribution of polyA tails.

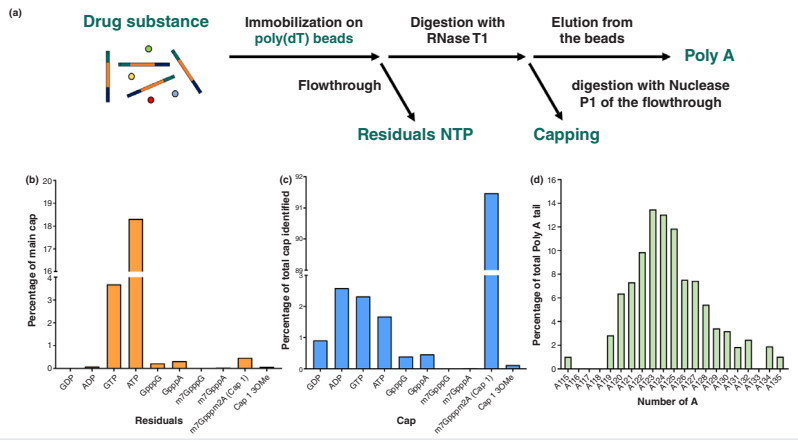
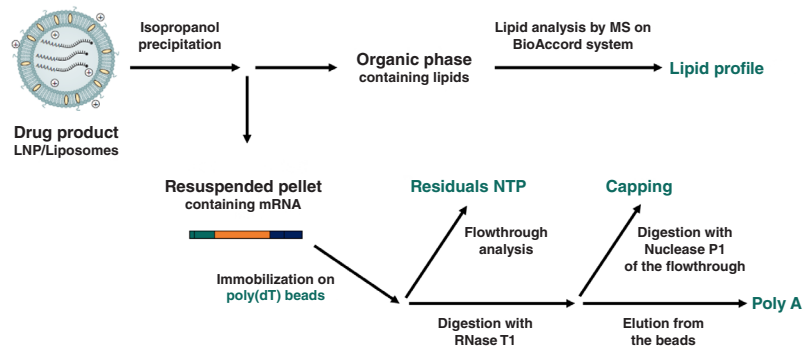


FIGURE 4: Workflow of the multiple attribute monitoring sample preparation and analysis of mRNA drug product



isopropanol (IPA), vortexed thoroughly and centrifuged 15 min at 14,000 g. The pellet is resuspended in RNAse-free water for further preparation steps.

Immobilization of mRNA on Poly-(dT) Beads: 4 µg of extracted drug product (DP) mRNA or 2 µg of Cas9 mRNA is hybridized with 200 µL of poly-d(T) immobilized on magnetic beads (RNA purification kit) during 10 min at room temperature. The flowthrough of this step is used for the analysis of the residual triphosphate nucleotides in the samples from the mRNA synthesis (using the LC–MS analysis method of 5'-cap)—as much volume as possible is injected to increase the probability to quantify residuals.

On-Bead mRNA Digestion: First digestion is performed using 1 µL of RNAse T1 and 19 µL of water during 2 h at 37 °C under agitation. Flowthrough is collected and further digested using 1 µL of Nuclease P1 and 1 µL of Nuclease P1 reaction buffer during 30 min at 37 °C under agitation (700 rpm). This digest is analyzed using LC–MS analysis of 5'-cap. Poly(A) tails are eluted by heating the beads at 70 °C in 22 µL of water during 8 min and the flowthrough is analyzed.

In-Solution Digestion of mRNA for 5'-Cap Analysis: 10 µg of Cas9 mRNA are digested using 1 µL of Nuclease P1 and 1 µL of Nuclease P1 reaction buffer, during 30 min at 37 °C under agitation (700 rpm). Enzyme is inhibited by heat inactivation at 70 °C during 10 min under agitation (700 rpm).

In-Solution Digestion of mRNA for 3'-Poly(A) Tail Analysis: 10 µg of Cas9 mRNA are digested using 1 µL of RNAse T1, during 30 min at 37 °C under agitation (700 rpm).

Preparation of 5'-Cap Standard Solutions: 5 µL of CleanCap AG were digested with 5 µL of Nuclease P1 and 5 µL of Nuclease P1 reaction buffer, during 30 min at 37 °C under agitation (700 rpm). All caps were prepared at 1 mM in water and mixed at equimolar concentration together (10 nM). Further dilutions were performed to inject between 1 fmol and 10 nmol of each cap.

LC–MS Analysis of 5'-Cap: Samples (10 µg for method development, 4 µg for DS and 2 µg for DP) are loaded on a Waters Premier BEH Oligonucleotides C18 (100 × 2.1 mm, 1.7-µm particles) and eluted using a 20 min gradient from 5% to 33.6% of solvent B (Solvent A: 5 mM dibutylammonium acetate – Solvent B: acetonitrile) at 0.2 mL/min, 30°C, on a Waters Acquity H-Class bio. Waters Xevo G2-XS QTof is coupled online with the UPLC system and is operated in multiple reaction monitoring (MRM) negative mode. Source parameters were set as follows: capillary voltage: 1.5 kV; cone voltage: 30 V; cone gas flow: 100L/h; desolvation gas flow: 500L/h; source temperature: 100 °C; desolvation gas temperature: 350 °C. Mass range was set to 50-4000 Th, scan time to 0.5 s and fragmentation energy (high energy ramp) from 20.00 to 40.00 eV.

LC–MS Analysis of 3'-Poly(A) tail: Samples (10 µg for method development,

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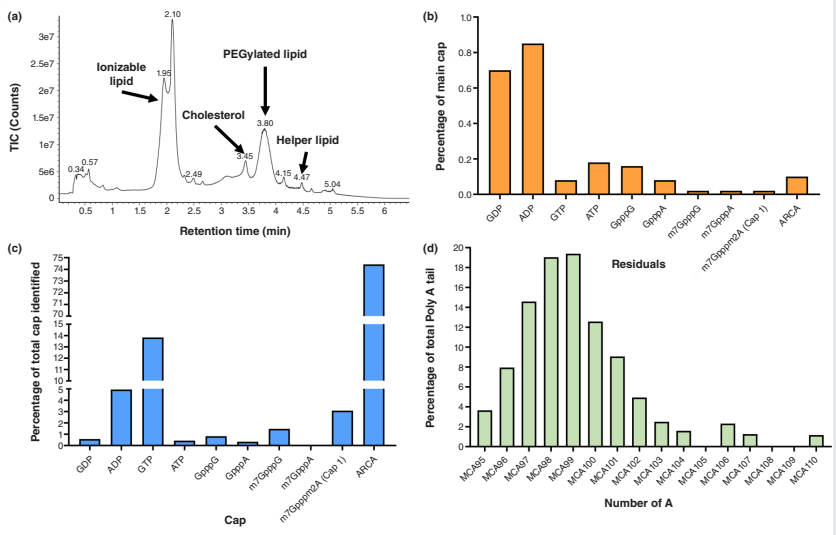
FIGURE 5: Analysis of a lipid-nanoparticle encapsulated mRNA. (a) Identification of lipids composing the lipid nanoparticle. (b) Relative quantification of residuals. (c) Relative quantification of cap. (d) Size and abundancy distribution of polyA tails.



4 µg for DS and 2 µg for DP) are loaded on an Agilent PLRP-S 1000Å (50 × 2.1 mm, 5-µm particles) and eluted using a 10 min gradient from 15% to 45% of solvent B (Solvent A: 15 mM dibutylamine, 25 mM hexafluoroisopropanol in water – Solvent B: 15 mM dibutylamine, 25 mM hexafluoroisopropanol in methanol) at 0.4 mL/min, 80°C, on a Waters Acquity H-Class bio. Waters Xevo G2-XS QTof is coupled online with the UPLC system and is operated in negative MS^E mode. Source parameters were set as follows: capillary voltage: 2.5 kV; cone voltage: 150 V; cone gas flow: 200L/h; desolvation gas flow: 500L/h; source temperature: 100°C; desolvation gas temperature: 350 °C. Mass range was set to 400–4000 Th, scan time to 1 s and fragmentation energy (high energy ramp) from 20.00 to 45.00 eV.

LC–MS Analysis of Lipids: Samples are loaded on a Waters Premier CSH Phenyl-Hexyl (50 × 2.1 mm, 1.7-µm particles) and eluted using a 6 min gradient from 60% to 90% of solvent B (Solvent A: 0.1% formic acid in water – Solvent B: 0.1% formic acid in ACN) at 0.4 mL/min, 50 °C, on a Waters BioAccord system (13). The system was operated in positive mode using an acquisition range of 50–2000 Th. Capillary voltage was set to 1.5 kV, cone voltage to 30 V with a fragmentation cone voltage of 100–150 V, a desolvation temperature of 350 °C, and a scan rate of 1 Hz.

Data Processing: Data processing is performed using UNIFI integrated identification and quantification modules. Poly(A) tailing deconvoluted spectra are further processed using homemade python script. Capping analysis was performed by extracting signal for each cap in both samples and calibration standard solutions. Generation of calibration curves was performed automatically within UNIFI and were used to correct and determine concentrations of cap.

Results

Development of Methods: Analytical methods were developed and refined using a commercially available Cas9 mRNA as the test subject. The manufacturer specifies that this mRNA is co-transcriptionally capped, resulting in the presence of Cap1 (m7GpppAm2) structures. However, no information was provided concerning the length of the poly(A) tail.

For capping analysis, Nuclease P1 was used in the sample preparation stage because it cleaves all phosphodiester bonds involving only one phosphate group, leaving the triphosphate of the cap intact. To achieve effective separation of various cap species from nucleotide monophosphates (NMPs)—the most abundant molecules produced post-digestion—a di-butylaminoacetic acid (DBuAA) gradient was used. As depicted in Figure 1a and 1b, this gradient successfully segregated all potential cap species from NMPs. Using cap standard curves and UNIFI's automated process, mRNA sample cap abundancies were computed and capping percentages quantified. The analysis revealed that 90.2% of the Cas9 mRNA was capped with Cap1 (Figure 1c), a result consistent with expectations based on the co-transcriptional capping method employed by the manufacturer.

To assess the distribution of poly(A) tail lengths, the approach relied on the use of RNAse T1, which cleaves at the 3' end of guanine (G) nucleotides. This enzymatic reaction generated short oligonucleotides and poly(A) tail, which were then subjected to LC–MS analysis. Due to limited chromatographic resolution for long oligonucleotides under these conditions, all poly(A) tails were visualized under a single chromatographic peak. This simplified the mass spectrometry data processing, because it required the analysis of only a single mass spectrum (Figure 2a). Deconvolution of the obtained mass spectrum (Figure 2b) allowed the determination of the molecular weights of the various poly(A) tails present (Figure 2c). An automated Python script was developed and utilised for peak

identification and poly(A) tail length calculations. Data analysis revealed that the most abundant poly(A) tail was A123, which accounted for 13.1% of the total population, as shown in Figure 2d.

Analysis of a Drug Substance: Recognizing that sample availability can often be a constraint, especially during early developmental stages, and given the high demand on mass spectrometers and technical personnel, the efficiency of sample preparation was sought to be improved and the time commitment required for experiments reduced. The optimized methodology minimizes both the sample volume and the overall time needed for the comprehensive analysis of the two critical quality attributes (CQAs) of interest compared to the independent experiments.

This enhanced strategy employs poly(dT) beads to capture mRNA through the hybridization of adenosine residues in the mRNA tail with thymidine residues on the beads (Figure 3a). The supernatant, which may contain residual nucleotides co-purified with the mRNA, can be examined using a technique similar to cap LC–MS analysis. During this stage, mRNA is anchored to the beads, allowing for digestion by RNase T1 into smaller fragments, including one carrying the cap, that are then released into the supernatant. Importantly, the poly(A) tails remain undigested and attached to the beads.

The uncaptured fraction was analyzed to identify any residual nucleotides that were not incorporated into the mRNA during synthesis or removed during purification. For the Cas9 mRNA, high levels of residual ATP were detected (18% of the main cap intensity) as well as 3.7% of residual GTP (Figure 3b). The capping analysis validated the initial findings, showing that 91.5% of the mRNA was capped with Cap1, a result closely aligned with the 90.2% observed in the preliminary experiments performed in the previous section (Figure 3b). However, an increase in ADP abundance was noted (from 0.2% to 2.6%), which can only be explained by a low abundance resulting in a wrong quantification, and a decrease in GTP (from 4.4% to 2.3%), which can be explained by the potential residuals that have been removed thanks to the mRNA immobilization on beads. The poly(A) tail analysis mirrored the initial results, identifying the most abundant poly(A) tail as A123, accounting for 13.5% of the population compared to 13.1% in the tests performed during method development (Figure 3d).

This refined approach significantly streamlines the characterization of mRNA CQAs by reducing sample size requirements and optimizing the use of mass spectrometers and personnel. Moreover, the time spent on mass spectrometry analysis can be further optimized since the mobile phases are compatible. However, this does necessitate the use of a quaternary pump coupled with a column manager and column selector to facilitate the simultaneous overnight analysis of both types of experiments.

Application of the Method to a Lipid-Nanoparticle Encapsulated mRNA: A distinguishing feature of mRNA-based drug products is their encapsulation within lipid nanoparticles. Due to this unique formulation, direct capture of mRNA on beads is infeasible, necessitating an additional extraction/precipitation step. Following extensive testing with various organic solvents, isopropanol precipitation was chosen as method of choice. This two-step procedure is both rapid and convenient, and avoids the use of hazardous solvents. After precipitation and solvent removal, the mRNA is reconstituted in water and immobilized on poly(dT) beads, following the same workflow used in drug substance analysis (Figure 4).

This optimized protocol was then applied to a lipid nanoparticle-encapsulated mRNA, thereby validating the methodology on an actual drug product. It is worth noting that the required sample amount is larger than what would typically be used for drug substance analysis, with at least twice the amount required prepared. This can be explained by an incomplete precipitation process, resulting in a reduced mass spectrometry signal. However, any additional prepared sample can be repurposed for other types of assays, such as purity analysis via capillary gel electrophoresis (cGE) or anion-exchange chromatography with UV detection (AEX-UV).

The sample preparation workflow yielded four distinct fractions: lipidic, residual, cap, and poly(A), each of which was analyzed using its respective method. The lipidic fraction revealed the presence of the four anticipated lipid types constituting the liposomal formulation: an ionizable lipid, cholesterol, a PEGylated lipid, and a helper lipid (Figure 5a). High concentrations of this lipidic fraction can also be injected to scrutinise potential impurities and degradation products.

In the residual fraction analysis, low-abundance species were detected, suggesting that the mRNA was encapsulated using a highly purified drug substance (Figure 5b). Capping analysis identified ARCA (Anti-Reverse Cap Analog) as the dominant cap, accounting for 74.4% of the total population (Figure 5c). Elevated levels of GTP (13.8%) were also detected. This is likely attributable to the post-transcriptional capping process, which involves multiple enzymes and reaction intermediates, suggesting the reaction may be incomplete.

Finally, spectral data processing for poly(A) tail analysis revealed MCA99 as a predominant species, which contains 99 adenosine residues along with two

additional residual nucleotides (i.e., N(1)-methylpseudouridine and cytosine). However, their sequential arrangement could not be determined (Figure 5d).

Conclusions

The methodologies developed offer comprehensive characterisation of two essential quality attributes of mRNA, demonstrating versatility because they can be applied to a wide range of mRNA types without any a priori conditions. The optimized workflow seamlessly integrates the analysis of both drug substances and drug products while minimizing sample requirements, sample preparation effort, and analysis time. This streamlining is of particular importance given the often-limited availability of sample quantities and the high demand for mass spectrometry resources. On-bead digestion does not appear to have any artefactual downsides, as the results obtained during method development and drug substance analysis are highly comparable with regard to Cap1 quantification. Only minor increases are observed for ADP and GDP, but these are correlated with a decrease in the amounts of ATP and GTP quantified. Poly(A) tail analyses are also consistent, with equivalent distributions observed in both experiments, which is not aligning with observations made by Strezscak et al. (14). However, unlike their experiment, the sample amounts used in our study are higher. This might explain the lack of observable effect of the beads on the poly(A) distribution.

Crucially, with a single sample preparation, these methods enable not only the assessment of poly(A) tail length and capping, but also the identification of residual nucleotides and the characterisation of the lipids constituting the lipid nanoparticles. This multi-faceted analysis, achievable with minimal sample input and preparation, further underscores the efficiency and comprehensive nature of the techniques developed in this study. In a context where there is a need for streamlined assessment of critical quality attributes, the multi-attribute approach offers great perspectives, by providing high quality results from a limited sample amount, using a single sample preparation and efficient LC–MS methods. This improves time-to-result while minimizing human resources and equipment resources.

This approach can be flexibly extended to integrate additional sample preparation steps or alternative analytical techniques. For instance, the proportion of polyadenylated mRNA could be accurately gauged by comparing bead-purified fractions against the flowthrough, thereby enabling the assessment of an even broader range of critical quality attributes for mRNA-based therapeutics. The methods outlined in this study not only offer robust solutions for current challenges but also provide a foundation for future advancements in the analytical characterization of complex mRNA-based therapeutic systems.

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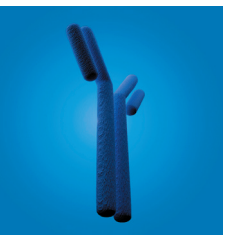
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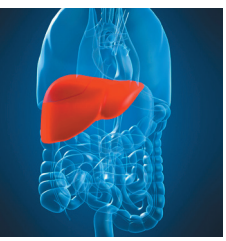
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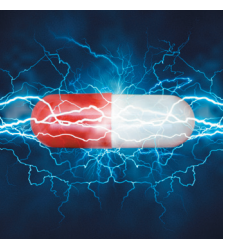
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Establishing Analytical and Functional Comparability for Biosimilars

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A *biosimilar* is a drug product that has been deemed to be highly similar to its off-patent reference product in terms of purity, molecular structure, and bioactivity. Approvals to such products are granted on the basis of unambiguous demonstration of no clinically meaningful differences between the reference and the intended biosimilar. For a successful biosimilar approval, establishing analytical and functional biosimilarity across all relevant critical quality attributes is an essential prerequisite. This critical activity is performed using a combination of orthogonal, high-resolution tools that can accurately quantitate the minor differences that exist. In this article, we review key findings from some of the recent biosimilarity assessments that we have published on biosimilars of granulocyte-colony-stimulating factor (G-CSF), insulin glargine, rituximab, and trastuzumab.

Biosimilars are defined as biotherapeutic drugs that have been shown to have a highly similar quality, safety, and efficacy profile to the original product. Fuelled by the patent cliffs, the focus of the biopharmaceutical industry has rapidly shifted towards production of biosimilars. For a successful biosimilar approval, establishing analytical and functional biosimilarities across all relevant critical quality attributes (CQAs) is an essential regulatory prerequisite. Unlike conventional small molecule drugs, biosimilars exhibit high complexity at the molecular level. Therefore, slight variations during the manufacturing of these complex protein molecules may lead to significant changes in the safety and efficacy profile of the therapeutic product. Biosimilarity exercises conducted towards this end are resource-intensive, requiring the use of multiple biophysical techniques that are sufficiently able to map the CQAs in comparison with the reference product (1). Here, we briefly describe the analytical and functional biosimilarity assessment platform and review key findings from some of the recent published assessments. The work highlights comparability across marketed Indian biosimilars while emphasizing the utility of a well-designed analytical platform sensitive enough to tease apart the variation present with respect to the CQAs (Table 1). The twofold purpose of these biosimilarity exercises is to perform a quality evaluation of the marketed products in the public forum for uptake by practitioners and patients alike, and to serve as a feedback mechanism for the regulators on the rigorousness of the regulatory approval process.

Non-Monoclonal Antibody (mAb) Products

Case Study 1: G-CSF (2014): Filgrastim, or recombinant methionyl-granulocyte colony stimulating factor (rG-CSF), is a glycoprotein (18.8 KDa) that significantly impacts proliferation and differentiation of cells of hematopoietic lineage. It is licensed for reducing the incidence and duration of post-chemotherapy neutropenia in patients with nonmyeloid malignancies and for mobilizing hematopoietic progenitor cells in transplantation patients (2). The innovator product is sold under the brand name Neupogen and is manufactured by Amgen. The patents on Neupogen

TABLE 1: Strategy for analytical and functional similarity assessment studies conducted at the Center of Excellence for Biopharmaceutical Technology (COE-CBT)*

Substance	Innovator	Patent Cliff	Biosimilars Studied	Characterization Tools	
GCSF	Neupogen (Amgen)	December 2013 (US), 2006 (Europe)	Emgrast (Gennova Biopharmaceuticals Ltd), Lupifil (Lupin Pharmaceuticals), Colstim (Zydus Cadila), Neukine (Intas Biopharmaceuticals), Grafeel (Dr. Reddy's Laboratories)	Primary structure (intact mass, peptide mapping)	LC–ESI-TOF-MS
				Secondary structure	CD spectroscopy
				Mass/size	SDS-PAGE
				Product related variants and impurities	Aggregation: SE-HPLC, oxidized/reduced variants: RPLC, oxidation: peptide mapping
				Potency	*In-vitro* cell proliferation
Insulin Glargine	Lantus (Sanofi)	2014	Glaritus (Wockhardt Ltd), Basaglar (Eli Lilly), Basugine (Lupin), and Basalog (Biocon)	Primary structure	LC–ESI-TOF-MS
				Higher order structure	CD
					FT-IR
					Fluorescence
				Product-related variants	SE-HPLC
					RP-HPLC
					ThT fluorescence
				Stability	SE-HPLC
					RP-HPLC
				Drug content	RP-HPLC
				Excipient estimation	AAS
					RP-HPLC
				Functional characterization	Glucose uptake assay (EC50)
Rituximab	Ristova (Roche)	September 2016 (US), November 2013 (EU)	Reditux (Dr. Reddy's Laboratories), RituxiRel (Reliance Life Sciences), Mabtas (Intas Biopharmaceuticals), Maball (Hetero drugs), Cytomab (Alkem Laboratories)	Primary structure (Intact mass, peptide mapping, N-glycan profiling)	LC–ESI-TOF-MS, QTOF-MS after procainamide labelling
				Higher order structure	CD, FT-IR, fluorescence
				Size heterogeneity	SDS-PAGE, SE-HPLC, DLS
				Charge heterogeneity	CEX-HPLC
				Functional characterization	FACS, ADCC, CDC, SPR
Trastuzumab	Herclon (Roche)	June 2019 (US), July 2014 (EU)	Trasturel (Reliance Life Sciences), Canmab (Biocon), Vivitra (Zydus Ingenia), Hertraz (Mylan)	Primary structure (intact mass, reduced mass, peptide mapping, PTMs, glycosylation profile)	LC–ESI-TOF-MS
				Higher order structure	FT-IR, CD, fluorescence spectroscopy
				Size heterogeneity	SE-HPLC, DLS
				Charge heterogeneity	CEX-HPLC
				Functional characterization	SPR, cytotoxicity assay, LDH assay, FACS

***Abbreviations:** CD - circular dichroism, FT-IR - Fourier transform infrared spectroscopy, HPLC - high performance liquid chromatography, MS - mass spectrometry, CEX - ion exchange chromatography, CE - capillary electrophoresis, RP - reversed-phase (liquid chromatography), SEC - size-exclusion chromatography, SDS-PAGE - sodium dodecyl sulfate poly acrylamide gel electrophoresis, SPR – surface plasmon resonance, ADCC - antibody-dependent cellular cytotoxicity, CDC - complement-dependent cytotoxicity, LDH - lactate dehydrogenase, FACS - fluorescence-activated single cell sorting.

These unbiased academic studies serve as a checkpoint for ensuring the efficacy of the regulatory approval process and also as a confidence building exercise in the manufacturing capability of organizations falling within a specific jurisdiction in developing efficacious biosimilar products.

expired in the United States in December 2013 and in Europe in 2006 (3). With Neupogen as the standard, biosimilarity assessment was undertaken for five marketed biosimilars (Emgrast from Gennova Biopharmaceuticals Ltd, Lupifil from Lupin Pharmaceuticals, Colstim from Zydus Cadila, Neukine from Intas Biopharmaceuticals, and Grafeel from Dr. Reddy's Laboratories) manufactured and approved in India. The analytical and functional platform used is as tabulated above (Table 1). Although an overall similarity was observed amongst the biosimilar products, the analytical strategy sensitively identified the presence of an atypical conformational variant present in three of the biosimilars through reversed-phase liquid chromatography (RPLC) and fluorescence detection (FLD) (2). Presence of a similar variant has previously been reported as an oxidized species.

Case Study 2: Insulin Glargine (2021): Insulin glargine is the first basal long-acting insulin analogue (6063 Da) produced in *Escherichia coli* (*E. coli*) by recombinant DNA technology. Its aqueous solubility is low at the physiological pH level, resulting in delayed absorption and a slower release (4). The innovator product is sold under the brand name Lantus and is manufactured by Sanofi. Patents on Lantus expired in 2014 in both the United States and the European Union (EU) (5). We evaluated the biosimilarity of four marketed insulin glargine products: Glaritus (Wockhardt Ltd), Basaglar (Eli Lilly), Basugine (Lupin), and Basalog (Biocon) across relevant CQAs through molecule specific analytical and functional characterization (see Table 1).

Although an overall structural and functional similarity was observed across insulin glargine biosimilars with respect to the innovator product, low amounts of product-related variants were seen in some biosimilars with a potential to impact product stability if found in higher amounts. Biosimilar 4 exhibited a higher content of high-molecular-weight species (HMWs) and related substances (RS) in comparison with the other products. Biosimilars 1 and 3 exhibited a higher rate of impurity generation per week. Furthermore, the percent aggregation in accelerated stability studies was found to statistically correlate (R^2 = 0.99, root mean square error [RMSE] = 0.095) with the percent aggregation at 0 days and the number of months from expiration, highlighting the overarching impact of the latter. In this study, based on available spectroscopic and chromatographic data and assisted by statistics, an order of biosimilarities was denoted to the products in the following order: Lantus, biosimilar 2, biosimilar 4, biosimilar 1, and biosimilar 3.

Monoclonal Antibody (mAb) Products

Case Study 3: Rituximab (2018): Rituximab was the first monoclonal antibody (mAb) approved for cancer treatment (B cell lymphoma) and was subsequently approved for immune-mediated and inflammatory diseases (such as rheumatoid arthritis and Wegener's granulomatosis). It is an IgG1k chimeric mAb (~145 KDa) produced in Chinese hamster ovary (CHO) cells and targets the B-cell surface receptor CD20 (6). The innovator is sold under the brand name Ristova and is manufactured by Roche. The patent on rituximab expired in September 2016 in the United States and in November 2013 in the EU (3). Since then, several biosimilars for the blockbuster drug have populated the marketplace. Biosimilarity assessment for rituximab included a comparison across five available marketed biosimilars: Reditux (Dr. Reddy's Laboratories), RituxiRel (Reliance Life Sciences), Mabtas (Intas Biopharmaceuticals), Maball (Hetero drugs), and Cytomab (Alkem Laboratories). One of the highlights of this study was utilizing procainamide labelling for quantifying the released glycans of the biosimilar products. As opposed to glycan profiling for the intact or reduced molecule, the information gained through labelling post-glycan detachment from the peptide backbone allowed for the quantitation of monosaccharides, such as sialic acid and other low abundance glycans. Biosimilar 2

was found to be the most similar with Ristova. Although G0F (55.8% vs. 26.1%) was the most abundant glycoform present in Ristova and most biosimilars, G2F (26.4% vs. 6.9%) was the most abundant glycoform in biosimilar 3. Biosimilar 3 also possessed a high percentage of galactosylated glycans (26.4% vs. 6.9%) and a low percentage of sialylated glycans (3.1% vs. 0%). Biosimilar 1 contained a higher amount of mannose group (3.1% vs. 0%) and afucosylation (2.4% vs. 0.6%) compared with the innovator Ristova. The analytical characterization platform also explored inter-product variability with respect to size (biosimilar 3) and charge variants (all except biosimilar 2) (6).

Case Study 4: Trastuzumab (2020): Trastuzumab is a humanized IgG1k mAb (145.53 KDa) directed against an epitope on the extracellular juxta membrane domain of the antihuman epidermal growth factor receptor (HER2). It is licensed as a therapy primarily for treating metastatic and early HER2-positive breast cancer. It was later indicated for HER2-overexpressing metastatic gastric cancer (7). The innovator product for trastuzumab is sold under the brand name of Herceptin (Herclon in India) and manufactured by Roche. The patent for the trastuzumab innovator expired in June 2019 in the United States and in July 2014 in the EU (3). Biosimilarity assessment consisted of four marketed biosimilars: Trasturel from Reliance Life Sciences, Canmab from Biocon, Vivitra from Zydus Ingenia, and Hertraz from Mylan (8). Through the correlation of structural deviations observed with product efficacy and potency (functional characterization), this study highlighted the structure-to-function relationship in biotherapeutics. Compared with the innovator, deviations observed in glycosylation, size, and charge variants consistently translated into reduced potency as measured through antibody-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) assays as observed in the case of biosimilar 2 (8).

Unlike conventional small molecule drugs, biosimilars exhibit high complexity at the molecular level. Therefore, slight variations during the manufacturing of these complex protein molecules may lead to significant changes in the safety and efficacy profile of the therapeutic product.

Conclusion

A well-designed analytical and functional similarity assessment plays a foundational role in the biosimilar approval process. As such, these studies need to be designed in accordance with the regulatory requirements of the jurisdiction for which the product is intended. Such unbiased academic studies also serve as a checkpoint for ensuring the efficacy of the regulatory approval process and also as a confidence building exercise in the manufacturing capability of organizations falling within a specific jurisdiction in developing efficacious biosimilar products.

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Critical Thinking On Characterization

Guest editors Valentina D'Atri from the University of Geneva in Switzerland and Jelle De Vos from the Research Institute for Chromatography (RIC) in Belgium, interviewed leading luminary of biopharmaceutical analysis, Reed Harris on critical quality attributes (CQAs), contemporary trends in biopharmaceutical characterization, and how he earned the moniker "Dr. Doom."

Reed Harris retired in 2022 after a 39-year career in biotechnology, starting as a research assistant and finishing as a senior staff scientist in the Roche-Genentech Pharma Technical Development department, with strategic, review, and teaching responsibilities. His former roles includ: Head of Investigational Medicinal Product Quality for Roche, senior director of Analytical Development & QC, analytical characterization scientist for Herceptin and Xolair, and head of a protein structure lab. He was the global (Genentech + Novartis) Xolair technical development team leader from Phase 2 through licensing. Reed is a CASSS Distinguished Fellow and has been an author of 49 publications, including 14 as corresponding author. He was a leader in the development of Roche-Genentech's strategies for post-approval comparability exercises, clinical and commercial specifications, the identification of critical quality attributes, linking structure to function, the use of a risk-based Quality by Design approach for control strategy development, unexpected protein modification identification, and curiosity-based thinking.

Jelle De Vos: Chromatographic methods can detect structural variants with altered function to allow the identification of critical quality attributes (CQAs). Ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), and size exclusion chromatography (SEC) are the key separation techniques, however, the link between the monoclonal antibody (mAb) structural changes and the effect on drug bioactivity is not always evident. How complicated is the process to determine what this link is?

It should not be too complicated. Chromatographic methods provide opportunities to collect intact material found in peaks or regions for molecular characterization and biological assay testing, such as potency testing and Neonatal Fc-receptor (FcRn) binding, which is relevant for pharmacokinetics. There is one caveat: The fractions collected from IEC and HIC methods should also be reanalyzed by SEC to determine if aggregated forms are enriched because aggregated forms may have an outsized effect on biological activity, thereby confounding the result interpretation. For IEC fractions, it is also useful to consider what the molecular charge differences will be at physiological pH 7.4; for example, a histidine residue that mediates chromatographic resolution when acidic mobile phases are used could be protonated during chromatography but unprotonated at physiological pH, so the physiological effects may be negligible.

Valentina D'Atri: The analysis of mAb charge variants by IEC can sometimes be challenged by the presence of a highly ordrered structure (HOS) acting as acidic forms. What could help to solve the puzzle?

Re-analysis of collected IEC fractions by isoelectric focusing, such as imaged capillary isoelectric focusing (icIEF), can help identify true charge differences. For example, deamidated, sialylated and proline amide forms will continue to be resolved by IEF. Conversely, HOS variation that gives an IEC separation due solely to charge presentation differences that affect interactions with the IEC column may not be resolved using icIEF. For example, an aspartate to isoaspartate change can affect charge presentation because the isoaspartyl modification rotates the polypeptide chain and extends the polypeptide chain due to incorporation of a methylene group, but the true charge difference between aspartate and isoaspartate side-chains is negligible,

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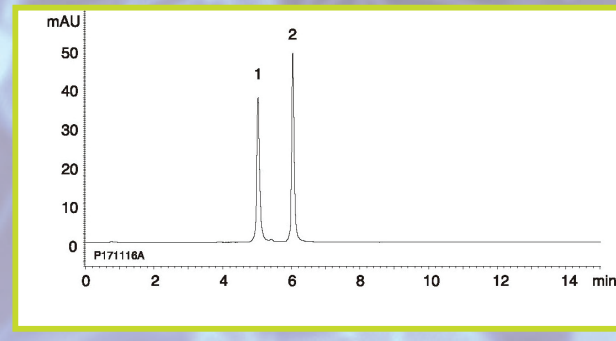
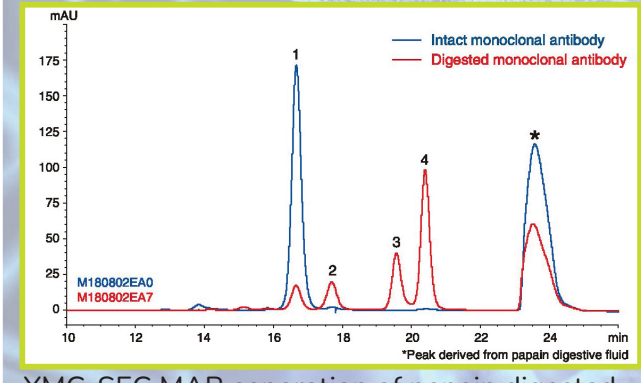


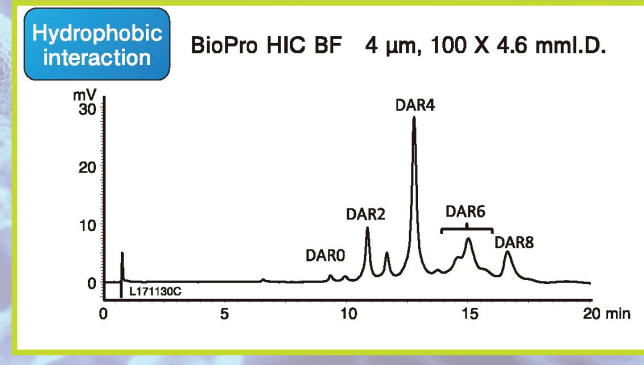
Separation of 20-mer and 21-mer oligonucleotides on BioPro IEX QF

mAB



YMC-SEC MAB separation of papain digested monoclonal antibody

HIC



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so modification impacts need to be evaluated based on the structural difference, not the charge difference.

> **Re-analysis of collected IEC fractions by isoelectric focusing, such as imaged capillary isoelectric focusing (icIEF), can help identify true charge differences.**

De Vos: Peptide mapping used as a multi-attribute method (MAM) approach is gaining attention because it allows us to follow different CQAs What are the regulatory challenges linked to this approach?
Health authorities worry about undetected variation that adversely affects product quality in ways that compromise patients. MAM is an excellent tool for monitoring multiple covalent modifications and variants, so it can address concerns about unexpected (non-model) variation when new peak detection is included in the analysis. Most of the MAM technical issues have been addressed, including sample preparation, chromatographic, ionization, and the use of reported (indicator) sites, as reviewed in Feng Yang's 2023 mAbs article (1). MAM also has some liabilities that need to be acknowledged. Thiol modification information will be lost if the peptide mapping step is performed after reduction and modification. Deamidation at an ADCC-relevant site in the heavy chain may not be detected because this is found in a small Val-Ser-Asn-Lys peptide peptide that may not be resolved from the void (salt) flowthrough, requiring alternative RP-HPLC columns or additional enzyme digests. Modification distribution information is also lost by the peptide mapping steps; for example, heavy chain Asn297 afucosylation symmetry is important because only one heavy chain needs to be afucosylated to affect in vitro antibody-dependent cell-mediated cytotoxicity, so glycopeptide analysis using MAM may need to be complemented with methods that report distribution.

An analytical package should include targeted attribute quantitation where degradation-susceptible sites are studied in stability studies and during process monitoring along with some intact or fragment chromatographic methods to cover gaps and detect higher-order structural variants.

> **MAM is an excellent tool for monitoring multiple covalent modifications and variants, so it can address concerns about unexpected (non-model) variation when new peak detection is included in the analysis.**

D'Atri: Can you describe how the development and characterization of mAbs transformed the life of analytical scientists in the latest years?
I entered this field with the false perception that the health authorities were adversaries. When I started interacting with reviewers and assessors, I realized that they are outstanding scientists responsible for protecting human health; they should be skeptical (trust but verify), and we need to provide them with the information they need to make important decisions. Some of the best experiences in my career were when I worked with health authority scientists and leaders, explaining our knowledge and approaches, and then incorporating their feedback into our strategies and submissions. We need to acknowledge the roles that many organizations such as CASSS, the Parenteral Drug Association, and the Asia-Pacific Economic Cooperation forum have played in improving sponsor and health authority interactions.

De Vos: Talking about career opportunities and risks for analytical scientists, what event led you to earning the "Doctor Doom" honorific?
I kept finding unexpected and variable-level modifications in our first therapeutic antibody, which began to annoy teammates because these discoveries were exciting for the analytical scientists but created new work for others. For example, the identification of sequence variants caused by mutations introduced during transfection meant that we needed a different cell line. Identification of an asparagine residue that deamidated during cell culture production required better process control. I learned to describe new findings in a more sympathetic way. Despite this, I earned the unfortunate "Dr. Doom" honorific from a project team colleague, as I was the person

I sometimes see people draw out carbohydrate structures with all the linkages named when they only have the mass; the author can claim that the result is consistent with a proposed structure, but that's all they can claim unless they're also running tandem mass spectrometry (MS/MS) or other methods to get structural information.

who always brought the unwelcome news. I remain convinced that the ability to identify unexpected variation is important for the regulatory credibility of any analytical program.

De Vos: What do you regard as the most game-changing technological development (in relation to sample preparation/chromatography/mass spectrometry) during your career?
For technical development, peptide LC–MS (including data processing) provided huge gains in speed and sensitivity for protein characterization, especially for modification identification. I used to run a laboratory that did amino acid analysis and Edman protein sequence analysis, both of which have been made obsolete by peptide LC–MS capabilities. One caveat: It is important to maintain some discipline regarding what can be claimed with only mass information. I sometimes see people draw out carbohydrate structures with all the linkages named when they only have the mass; the author can claim that the result is consistent with a proposed structure, but that's all they can claim unless they're also running tandem mass spectrometry (MS/MS) or other methods to get structural information.

D'Atri: Do you believe that you witnessed the biggest changes within the biotechnology field throughout your career, or is the best yet to come?
That's a broad question. For therapeutic proteins, the basic process and analytical technologies are very mature now, which enables productivity optimization, but we have to continue to encourage curiosity and innovation. There are still unknowns and things that we should understand better. Leaders and scientists need to ask big questions — What causes immunogenicity? Why does this take so long? Is there a better way to get product quality information? How can we reduce process and testing variation? — and then give people time to work on those questions.

De Vos: What are your thoughts on the new modalities, such as gene therapy products, viral vectors, oligonucleotide drugs? Will these reshape the landscape of analytics just like when monoclonal antibodies made their mainstream introduction around 20 years ago?
The analytical strategy framework that we have used for therapeutic proteins will remain necessary for new modalities. This means deciding three things: What to measure, how to measure, and acceptable results. Biotechnology has also made it respectable for top-notch scientists and engineers to work in industry, so I expect that all this talent will drive rapid advances for new modalities.

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Valentina D'Atri holds a PhD degree in Industrial and Molecular Biotechnologies, and she is currently a Research and Teaching Fellow at the University of Geneva, Switzerland. Her interests and research activities focus on the development of cutting-edge LC–MS analytical workflows for the detailed characterization of innovative therapeutic drugs such as biopharmaceutical proteins, therapeu-tic oligonucleotides, and viral vectors. She has currently authored over 55 peer-reviewed contribu-tions including articles and book chapters.
Jelle De Vos currently works at the RIC group as an expert on the development of chromatography methodologies for the analysis of complex biopharmaceutical samples. He focuses on pushing the boundaries of separation science and has published over 30 articles on advancing the performance limits of ultrahigh-pressure liquid chromatography technology and the development of microfluidic technology for liquid chromatography

High-Throughput Monitoring of Biotherapeutic Critical Quality Attributes with High-Resolution Ion Mobility-Mass Spectrometry (HRIM-MS)

Steven Broome[1], Shahadat Reza[1], Harsha Gunawardena[2], Kelly L. Wormwood Moser[1], Brett Peterson[1], and Frances Carroll[1],

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Integrating HRIM into conventional LC–MS protocols enhances the reproducibility and efficiency of high-throughput targeted PTM analysis, significantly advancing biopharmaceutical characterization.

Post-translational modifications (PTMs) to peptides can disrupt secondary and tertiary protein structure, leading to changes in safety or efficacy of protein-based therapeutics. Monitoring of PTMs to ensure production and distribution of safe and efficacious biotherapeutics requires robust and high-throughput analytical techniques capable of detecting extremely small differences in structure. Traditional liquid chromatography–mass spectrometry (LC–MS) peptide mapping workflows are time-consuming and complex, resulting in delayed project turnaround timelines (TATs). We implemented HRIM technology into traditional LC–MS peptide mapping characterization workflows to perform rapid, targeted analysis of critical quality attributes (CQAs) that are often difficult to separate or potentially go undetected with platform LC–MS methods.

Materials and Methods

Data acquisition was carried out on a high-resolution ion mobility (HRIM) MOBIE® instrument (MOBILion Systems) coupled to a 6545XT QTOF (Agilent Technologies) with a 1290 Infinity II Autosampler (Agilent Technologies). Default peptide mapping LC–HRIM-MS method parameters were used. A 20-min LC gradient (Table 1) was employed using a 2.1 × 50 mm, 1.8 µm Agilent Zorbax Extend-C18 Rapid Resolution column HT. A flow rate of 0.4 mL/min was used with a 2 µL injection volume. The SLIM chamber was

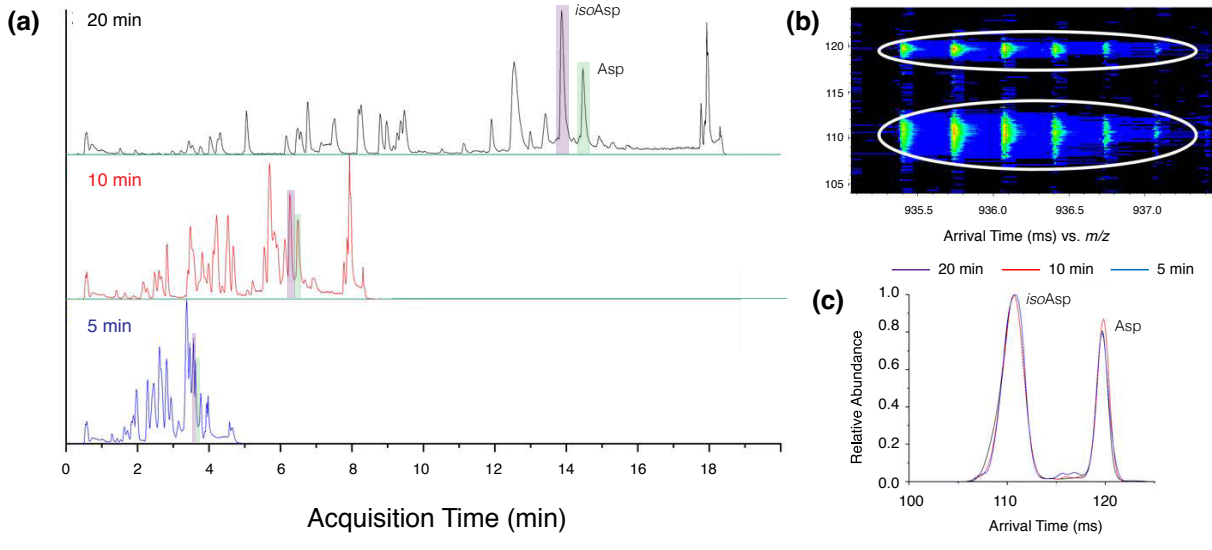

Figure 1: (a) Total ion chromatogram for 20-, 10-, and 5-min LC–HRIM-MS analysis of NIST mAb digest; (b) HRIM-MS heat map plot demonstrating mobility resolution of modified (*iso*Asp) and native (Asp) 24-residue peptides; (c) Overlaid extracted ion mobiligrams of the modified (*iso*Asp) and native (Asp) 24-residue peptides from each LC gradient used.

Table 1: 20-min LC gradient used for LC–HRIM-MS analysis. Buffer A: 0.1% formic acid in water, Buffer B: 0.1% formic acid in acetonitrile

Time (min)	%A	%B
0.00	99	1
16.00	65	35
17.30	60	40
17.40	0	100
17.90	0	100
17.91	99	1
20.00	99	1

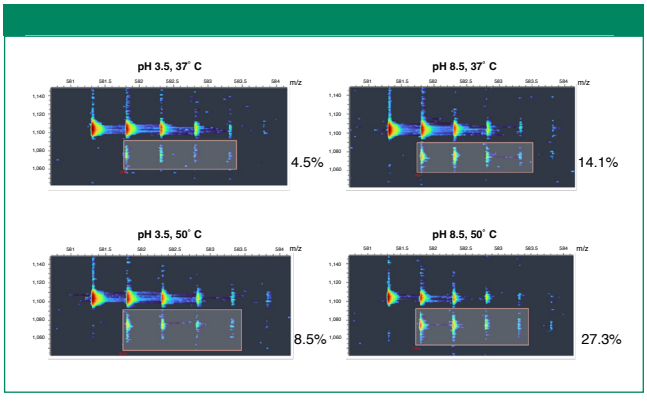

Figure 2: HRIM-MS heat map plots showing relative quantitation of modified andunmodified 2+ charge state NISTmAb HC 364-373 NQVSLTCLVK (*m/z* 581.25) at four different stress conditions.

maintained at 2.5 Torr throughout the experiments. Two 24 residue isomers containing aspartate (Asp) and iso-aspartate (*iso*Asp) forms were analyzed individually and as a mixture for HRIM method development. These standards were spiked into NIST mAb trypsin digest to simulate analysis in matrix.

Temperature- and pH-stressed NIST mAb trypsin digest samples were prepared and provided by collaborators. Data analysis and visualization was achieved using Protein Metrics Byos for HRIM Peptide Workflow.

Results

LC–HRIM-MS analysis of modified (*iso*Asp) and unmodified (Asp) peptide standards spiked into NIST mAb trypsin digest produced reproducible mobility separation of the peptide standards across multiple LC gradients ranging from 20- to 5-min, as shown in Figure 1. Baseline resolution of the isomerized peptide from its native form was achieved with consistent arrival times independent of the LC gradient used. This demonstrates the utility of LC–HRIM-MS for high-throughput targeted PTM analysis, specifically for distinguishing and monitoring Asp vs. *iso*Asp.

In addition, a forced degradation study assessing four different stress conditions was performed using the standard 20-min LC–HRIM-MS workflow to demonstrate the platform's ability to

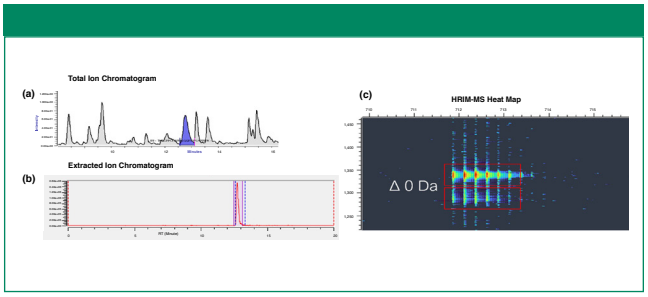

Figure 3: (a) Total ion chromatogram for 20-min LC–HRIM-MS analysis of NIST mAb digest; (b) Extracted ion chromatogram for 4+ charge state NISTmAb heavy chain peptide 226-249, THTCPPCPAPELLGGPSVFLFPPK (*m/z* 711.85); (c) HRIM-MS heat map plot demonstrating mobility resolution of potential isomers.

perform relative quantitation for target deamidated peptides. As displayed in Figure 2, relative quantitation based on the mobility features shows increasing percent modification with increasing temperature and pH, as expected. The Protein Metrics Byos for HRIM Peptide workflow was used for 4D feature finding and relative quantitation.

During this analysis, separation of a potential peptide isomer was observed in the mobility dimension as well (Figure 3). In this case, no separation was observed in the chromatographic dimension, demonstrating the value of adding HRIM to resolve potential PTMs not resolved by LC. Incorporating HRIM into traditional LC–MS workflows enables rapid relative quantitation of target PTMs.

Summary

This work demonstrates the benefit of incorporating HRIM in traditional LC–MS methods. With the highest single pass ion mobility resolution, the MOBIE™ platform allows for reproducible separation of select isobaric and isomeric PTMs (deamidation and isomerization) in the mobility dimension. The incorporation of Protein Metrics Byos for HRIM data analysis software provides a full workflow solution for rapid characterization of target PTMs by LC–HRIM-MS.

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Simultaneous LCMS Analysis of Ultra-Short Through Long Chain PFAS Compounds (C1-C10) in Industrial Water and Ground Water

Introduction

Shodex collaborated with Claros Technologies using a HILICpak™ VT-50 2D Shodex column to study per- and polyfluoroalkyl substances (PFAS) in industrial and ground water samples. PFAS have been in industrial use since the 1960s and are commonly found in the environment. However, PFAS compounds have been linked to multiple health concerns, including cancer, birth defects, and many other life-threatening health conditions. As a result, the Environmental Protection Agency (EPA) has put global PFAS advisories in place to drive the development of PFAS monitoring. Like long chain PFAS compounds, short-chain PFAS compounds do not easily break down in the environment and have high mobility in water and soil, leading to an increased focus on detecting ultra-short through long chain PFAS. The current challenge to overcome is that ultra-short chain and long chain PFAS cannot be simultaneously retained on a C18 column, as shown in EPA methods 533 and 537.1. A validated method was developed for screening short to long chain PFAS in industrial and ground water, using direct injection with no sample preparation. This method uses multimode chromatography coupled with mass spectrometry for increased sensitivity and detection of ultra-short chain to long length PFAS compounds.

Experimental

Instrument	Waters TQ-Absolute coupled with Acquity Premier UPLC
Column	Shodex HILICpak VT-50 2D (2.0 mm I.D. x 150 mm)
Solvent A (26%)	50 mM Ammonium Acetate (pH 6.8)
Solvent B (74%)	Acetonitrile
Flow rate	0.2 mL/min
Detector	ESI-MS MRM(-)
Column Temperature	40°C
Injection volume	5 µL
ESI voltage	0.5 kV
Cone voltage	Compound dependent
CE	Compound dependent
Source temperature	150 °C
Desolvation temperature	500 °C
Cone gas (L/Hr)	150
Desolvation gas (L/Hr)	1000
Nebulizer gas (L/Hr)	300

Results

Industrial and ground water samples from clients were studied for matrix effects on the analysis of ultra-short through long PFAS compounds by LC/MS. This method utilizes multimode chromatography with HILIC and anion exchange retention characteristics. For calibration standards in de-ionized water, most compounds reported an LOD of 0.05 ppb and a R^2 of 0.999, apart from TFA and TFMS. Using direct injection, without sample preparation, sub-ppb to ppt concentration levels for several PFAS species

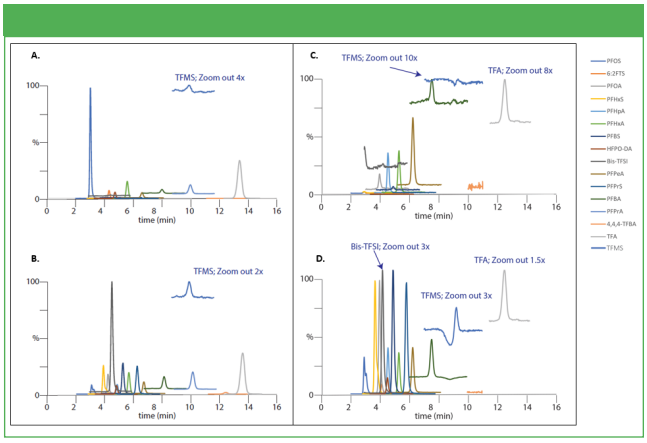

Figure 2. A) Direct injection MS analysis of industrial water. **B)** Industrial water MS analysis with 1ppb calibrant spiked. **C)** Direct injection MS analysis of ground water. **D)** Ground water MS analysis with 1ppb calibrant spiked.

were found to be indigenous to the industrial and ground water samples. The highest concentration PFAS compound found in industrial water was PFHxA (0.33 ppb), while in ground water, it was TFA (0.529 ppb). For accuracy evaluation, recoveries of 1-ppb spiked standards were under 10% in both matrices, apart from TFMS and 444-TFBA in ground water (~15%) likely due to an increase in interference from organic compounds. Across the spiked calibration range of 0.05 ppb to 100 ppb, the R^2 recovery was found to be at least 0.99 in both samples (Figure 1B & D).

Conclusion

The Shodex™ HILICpak™ VT-50 series, polymer-based quaternary ammonium type HILIC columns demonstrated highly selective and sensitive LC/ESI-MS measurements in industrial water and ground water without sample preparation or using ion-pair reagents. The chromatographic separation utilized a polyvinyl alcohol solid support with quaternary ammonium surface functional groups, the VT-50 series, capable of HILIC and anionic retention capabilities in buffered acetonitrile eluents. The simple isocratic mixture of 50 mM aqueous ammonium acetate solution and acetonitrile using multimode separation allows for the analysis of ultra-short through long chain PFAS in real world samples, which cannot be simultaneously retained using reversed phase methods.

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